

Glomerular Basement Membrane Antigens and Goodpasture's syndrome

Jörgen Wieslander



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Glomerular Basement Membrane Antigens
and
Goodpasture's Syndrome

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Abstract The aim of the present investigation was to isolate antigens from the glomerular basement membrane that could be used in assays of circulating anti-glomerular basement membrane antibodies and to purify those antigens involved in Goodpasture's syndrome. An ELISA was developed for the assay of anti-basement membrane antibodies and for the quantitation of antigenic components. The antigens studied were solubilized from glomerular basement membrane using proteolytic enzymes and chaotropic agents, respectively. Antibodies from patients with Goodpasture's syndrome react most strongly with antigens solubilized with collagenase, while antibodies from patients with other subsets of glomerulonephritis give strongest reaction with antigens extracted with guanidine-HCl. Antibodies in different forms of glomerulonephritis, then, appear to react with different antigenic structures, possibly of future diagnostic value. The antigen in Goodpasture's syndrome was further studied. It was isolated from collagenase digests of glomerular basement membrane and found to be a collagenase resistant polypeptide with a molecular weight of 26 000. The same antigen is also present in apparently dimeric molecules with a similar amino acid composition. A number of patients with Goodpasture's syndrome all had circulating antibodies to both antigenic components. The size and the amino acid composition of the purified antigen is similar to that of one of the terminal collagenase resistant fragments of type IV collagen. It could indeed be shown that the antigen is present in isolated type IV collagen and could be isolated after collagenase digestion.		
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LIST OF PAPERS

This thesis is based on the following papers referred to in the text by their Roman numerals.

- I. Human glomerular basement membrane. Heterogeneity of antigenic determinants.
Jörgen Wieslander, Per Bygren and Dick Heinegård.
Biochim. Biophys. Acta. 553, 244 - 254, (1979).
- II. Antibasement membrane antibody: Immunoenzymatic assay and specificity of antibodies.
Jörgen Wieslander, Per Bygren and Dick Heinegård.
Scand. J. Clin. Lab. Invest. 41, 763 - 772, (1981)
- III. Antiglomerular basement membrane antibody: Antibody specificity in different forms of glomerulonephritis.
Jörgen Wieslander, Per Bygren and Dick Heinegård.
Kidney Int. 23, 855 - 861, (1983).
- IV. Goodpasture's syndrome. Isolation of the specific glomerular basement membrane antigen.
Jörgen Wieslander, Per Bygren and Dick Heinegård.
Submitted to Proc.Natl.Acad.Sci.USA
- V. Goodpasture's antigen of the glomerular basement membrane: Localization to noncollagenous regions of type IV collagen.
Jörgen Wieslander, Per Bygren, Dick Heinegård, Jon F. Barr, Ralph J. Butkowski, Shelley J. Edwards and Billy G. Hudson. Manuscript.

ABBREVIATIONS

GBM	Glomerular Basement Membrane
ELISA	Enzyme Linked Immunosorbent Assay
EHS sarcoma	Engelbreth-Holm Swarm sarcoma

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BACKGROUND

Structure and function of GBM

Basement membranes are specialized forms of extracellular matrix, composed of collagenous and non-collagenous glycoproteins as well as proteoglycans. The membranes form a border between epithelial cells and connective tissue matrix and provide a substrate on which the cells grow.

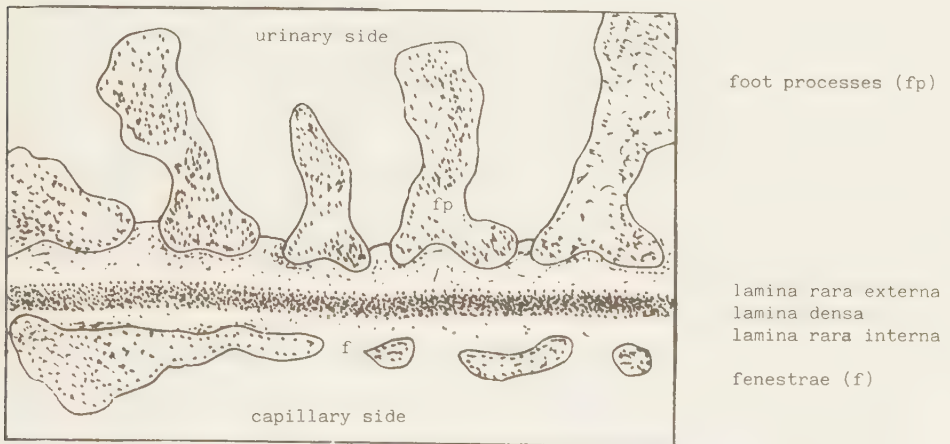


Figure 1. Diagram of GBM

A specialized and biologically important basement membrane is found in the glomerular capillary wall. On the capillary side the endothelial cells form a continuous sheet interrupted by pores called fenestrae (Fig. 1). The epithelial cells on the urinary side has characteristic foot processes separated by slits. Other parts of the membrane face the mesangial cells with their matrix. The cells synthesize the components of GBM and participate in repair processes (1). A major function of the glomerulus is to provide an ultrafiltrate of the blood plasma as the first step in the formation of urine. Proteins

with a high molecular weight and blood cells are retained in circulation, while low molecular weight molecules passes through to the urinary side. The filtration barrier is formed by GBM, which is the only continuous layer of the glomerulus (2). This molecular sieve excludes molecules larger than 70 000 (3). Furthermore, filtration is charge dependent and negatively charged molecules have restricted passage through the filter (4), probably due to fixed negative charges of GBM.

The glomerular basement membrane has several distinctive features (5). It is thicker (250 nm) than other basement membranes, being formed by fusion of endothelial and epithelial basement membranes. Like other basement membranes the GBM appears amorphous when studied by microscopy and does not show the typical fibrillar cross striated structures of interstitial collagens, although collagen is a major component. Three different layers can, however, be identified: the middle dense layer, called lamina densa and two layers of lower density, lamina rara interna and externa (Fig. 1). The lamina densa has been found to contain a network of fine filaments 3 nm in diameter (1), consisting mainly of collagen (5).

Preparation of GBM

In general it is difficult to prepare basement membranes free from adherent connective tissue (6). The only exception is the basement membrane of the lens capsule that can be isolated by mild mechanical means (6). The first method to isolate GBM was described by Krakower and Greenspoon (7). In their procedure kidney cortex was minced and the material that could be pressed through a fine mesh stainless steel screen was considered to represent glomeruli. The isolation procedure was modified by Spiro (8) who used several different sieves not only for

disruption but also for the separation of glomeruli from other tissue elements.

Isolated glomeruli are then sonicated (7,8) or cells are disrupted by treatment with detergents such as Tween 80 (9) or sodium deoxycholate (10,11) to separate the cells from the glomerular basement membrane. Several modifications of these methods have been introduced. Westberg and Michael (12) filtered the sonicated material through a fine sieve to remove insufficiently disrupted glomeruli and Freytag et al (13) included protease inhibitors during isolation. Both methods, however, have their disadvantages. Some authors (11,14) claim that detergents give a purer preparation with little risk of fragmentation of the molecules and with no loss of glycosaminoglycans (15). GBM prepared by detergent procedures has a high content of mesangial matrix (16). It has on the other hand been claimed that the heterogeneity of components seems to be the same whether sonication or detergent procedures are used (17,18) and that there is no loss of glycosaminoglycans upon sonication (18). Therefore, depending on the components under study, either procedure can be used.

Composition of GBM

GBM has a characteristically high content of proline, hydroxyproline, glycine and hydroxylysine (Table I). A large proportion of the total hydroxyproline is the 3-hydroxy isomer. The carbohydrate content is high (10%) and divided between two types of oligosaccharide that was first isolated by Spiro (19,20) from collagenase-pronase digests of GBM. One is a disaccharide unique to collagen (21), 2-O-D-glucosylgalactose linked by a beta-glycosidic bond to the hydroxyl group of hydroxylysine. The other is a complex type of oligosaccharide linked to asparagine. The amino acid composition as well as

the presence of glucosylgalactose points to the collagenous nature of the major GBM constituents.

Table I: Chemical composition of human GBM (from ref. 12)

	Residues per 1000 Amino Acid Residues		Residue Weight, g/100 g
3-Hydroxyproline	22.2	Sialic acids	0.88
4-hydroxyproline	81.3	Glucosamine	1.23
Aspartic acid	67.7	Glucose	2.78
Threonine	36.7	Galactose	3.00
Serine	48.9	Fucose	0.23
Glutamic acid	98.3	Mannose	0.57
Proline	57.9	Phospholipids	2.05
Glycine	221	Cholesterol	0.72
Alanine	59.8		
Valine	35.6		
Methionine	13.2		
Isoleucine	31.3		
Leucine	65.3		
Tyrosine	15.3		
Phenylalanine	24.9		
Hydroxylysine	26.1		
Lysine	19.5		
Histidine	14.2		
Arginine	38.0		
Half-cystine	22.8		

The glomerular basement membrane is essentially insoluble in physiological buffers because of the presence of multiple crosslinks. All cysteine residues appear to form such disulphide bonds, since free sulphydryl groups have not been detected (22). Additional stability is obtained by hydroxylysine derived crosslinks (23). Reduction of disulphide bonds in urea or SDS, however, results in solubilization of 80% of the membrane (22) and the release of a large number of polypeptide components which range in molecular weight from 30 000

to over 700 000 (24). The analytical data suggests the presence of both collagen like triple helical and globular domains on the same peptide chain (25).

Many of the problems in the isolation of components from glomerular basement membranes have been overcome by using the basement membrane producing EHS sarcoma (26) as a model system.

The main components isolated from basement membrane are listed in Table II. Included is the type IV collagen (27), the main component of basement membranes. Additional collagenous components are type V collagen (28) and intima collagen (type VI) (29). Examples of noncollagenous components are laminin (30), heparan sulphate proteoglycan (31), fibronectin (32), entactin (33) and amyloid P component (34).

Table II: Components of basement membranes

Collagens	Size	Composition	Size of chains	Proposed function
Type IV collagen	500 000	[pro α 1(IV)] ₃ [pro α 2(IV)] ₃ [pro α 1(IV)] ₂ [pro α 2(IV)] ₁	185 000 170 000	structural element
Type V collagen	300 000	[α 1(V)] ₂ [α 2(V)] ₁ [α 3(V)] ₃		
Intima collagen	160 000		40-50 000	
Noncollagenous components				
Laminin	1 000 000	AB ₃	A 450 000 B 220 000	cell adhesion proteins
Heparan sulphate proteoglycan	130 000	Protein core with heparan sulphate chains	GAG 25 000	negative charge barrier of BM
Fibronectin	450 000		220 000	cell adhesion protein
Entactin	158 000	contains sulphate		cell adhesion protein?
Amyloid P component	235 000		23 500	

Type IV collagen. Immuno-cytochemical studies have shown that type IV collagen is localized in the lamina densa of the GBM (35). Kefalides (36) isolated the collagen from GBM by pepsin digestion and suggested (37) that it consisted of a polypeptide chain with a size corresponding to that of an alpha-chain of interstitial collagens. Type IV collagen is now considered to consist of a large triple helical domain and a few non-collagenous segments, a structure referred to as procollagen like (Fig. 2). In the electron microscope the collagen is seen as thin strands, being 2-3 nm in diameter and up to 300 nm in length, with globular units at their ends (38). The latter resemble the noncollagenous terminal extensions of the soluble precursors of the interstitial collagens, i.e. the procollagens (39).

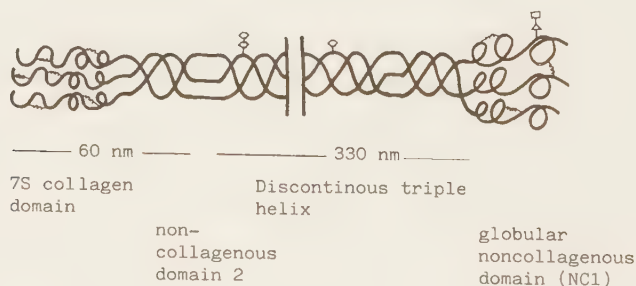


Figure 2. Procollagen like model of type IV collagen

It was proposed that the triple helical collagen from basement membrane has the molecular composition α -1(IV)₃ (27). This suggestion that GBM collagen contains only one type of alpha chain has proved to be an oversimplification since fragments of several types of chains have been detected in pepsin digests of GBM (40,41,42). Today it is known that there are two types of collagen chains namely α -1(IV) and

alpha-2(IV) having molecular weights of about 185 000 and 170 000, respectively (43,44,45,46,47,48) i.e. they are larger than the corresponding structures of interstitial procollagen. The chains are structurally distinct as shown by peptide mapping (49,50). Available data indicate that the composition of the type IV collagen with respect to the proportion and type of alpha-chains, varies with tissue as well as with species of origin (37,50,51,52,53). In contrast to the interstitial collagens, the type IV collagen is not cleaved to a smaller protein when incorporated in the tissue matrix (43,54,55). There are also other structural differences between type IV collagen and interstitial collagens (Table III). The type IV collagen has a higher content of hydroxylated amino acids and a lower content of alanine, arginine and glycine (27). The repetitive sequence Gly-X-Y, which forms the triple helical structure, may in certain positions have glycine substituted by other amino acids, with the result that the triple helical conformation is interrupted (56,57) resulting in increased sensitivity to proteases.

Carbohydrate accounts for around 10% of the weight of type IV collagen. Almost all of the hydroxylysine residues are glycosylated with glucosylgalactose disaccharides (19,20). Additional carbohydrates are oligosaccharide side chains linked to asparagine (58).

Table III: Characteristics of type IV collagen

1. Amorphous on EM. No cross striated fibrils observed.
 2. Collagen molecules of higher molecular weight than interstitial procollagens.
 3. The triple helical structure of type IV collagen is interrupted by short non triple helical sequences.
 4. No removal of terminal non-triple helical peptides when incorporated into the tissue.
-

A useful tool in the study of type IV collagen has been digestion using bacterial collagenase, which yields major fragments around 25 000 and 50 000 dalton (59,60) mainly containing the non-triple helical globular terminal peptides. The fragment having a molecular weight of around 25 000 (NC1 peptide) is probably derived from the C-terminal extensions of the type IV collagen molecule (61).

Another type of fragment, the 7S collagen, was first isolated from a collagenase digest of the EHS sarcoma (62). It has a molecular weight of 360 000 (63). Upon reduction it gives rise to a mixture of peptides ranging from 15 000 to 150 000 dalton (63). The 7S collagen is resistant to both collagenase and pepsin, possibly because of extensive crosslinking by disulphide bonds. Electron microscopy has shown that the 7S collagen represents a domain of type IV collagen in which 4 triple helical molecules are held together by disulphide bridges (64). It thus forms a junctional region in the type IV collagen network. The fragment can be identified in digests of most basement membranes (65).

Laminin. Laminin is a glycoprotein with a relatively high content of carbohydrate (15%) (66). It is present in all basement membranes although it was first isolated from the EHS sarcoma (30) and mouse carcinoma cells (66). The molecule is distinct from collagen and fibronectin with a very high molecular weight of about 10^6 . On reduction it yields two subunits with molecular weights of 220 000 and 440 000, respectively (30). The molecule can be visualized by electron microscopy as a cross with nonequal arms (67). Available data indicate that the 440 000 dalton subunit forms the long arm and three of the 220 000 dalton subunits form the short arms (68). Laminin is primarily found in the lamina rara, while absent or present

only in low concentrations in the lamina densa (69). The localization of laminin to the lamina rara is consistent with a function in the attachment of endothelial and epithelial cells to the GBM. Available data indicate that laminin mediates the attachment of epithelial cells to basement membrane collagen (70). Laminin can also bind to heparan sulphate and heparin (71).

Proteoglycans. Both heparan sulphate and hyaluronic acid have been isolated from GBM (72,73). More recently two types of intact proteoglycans have been purified from extracts of GBM (74), 85% by mass being a heparan sulphate proteoglycan and 15% a chondroitin sulphate proteoglycan. The latter is possibly derived from mesangial cells. They both had an estimated molecular weight of 130 000. The proteoglycans contain an estimated four to five glycosaminoglycan chains each with a molecular weight of about 25 000 (74). Other studies indicate, however, that the heparan sulphate chains have a molecular weight of only 13 600 (73). A heparan sulphate proteoglycan has also been isolated from the EHS sarcoma (31) although this proteoglycan has a much higher estimated molecular weight of about 700 000, with heparan sulphate chains of about 70 000.

The negatively charged heparan sulphate proteoglycan is assumed to be the factor responsible for the hindered passage of anions through GBM (75).

Fibronectin. Fibronectin is a major product of cultured fibroblasts (76) and has been shown to be almost identical to cold insoluble globulin present in serum (77). The molecular weight of fibronectin is 450 000. Its two subunits of 220 000 daltons each, are linked by disulphide bridges (78).

Fibronectin contains specific binding regions for a number of molecules such as fibrinogen/fibrin, collagen/gelatin, heparin and heparan sulphate, cell surface components and hyaluronic acid (79). It is considered that one of its functions is to provide cell attachment to matrices. Fibronectin is found associated with basement membranes, mainly in the mesangial areas. Whether or not it is present in the GBM itself is still unclear. Some studies using immunofluorescence indicate the presence of fibronectin (80), localized to lamina rara of GBM (80) while others report no staining for fibronectin (81). In view of its function in the attachment of endothelial and epithelial cells to the GBM it is likely that fibronectin is present in the lamina rara (5).

Organization of the GBM

The molecular organization of GBM has been an issue of some controversy and a number of different models have been proposed. Kefalides and coworkers (27) suggested that basement membranes contain a discrete collagen (type IV) having short noncollagenous extension peptides, which can be removed by digestion with pepsin. The collagen chains are linked to high molecular weight glycoproteins via disulphide bonds involving the extension peptides, thereby forming a network. Spiro and coworkers (82) proposed on the other hand that GBM consists of a network of disulphide-linked polypeptides each different in size and relative content of collagen-like sequences.

The discovery of 7S collagen as the crosslinking domain of type IV collagen (64) as well as the identification of the NC1 globular domain and the application of the rotary shadowing technique (83) in the study of type IV collagen, has allowed Timpl and coworkers (60) to construct a new model of type IV

collagen (Fig. 3). In this model four collagen molecules are joined by disulphide bonds in the 7S terminal region. Two collagen molecules are also connected to each other in the other terminal domain (NC1) giving rise to a network similar to that of a chicken wire fence.

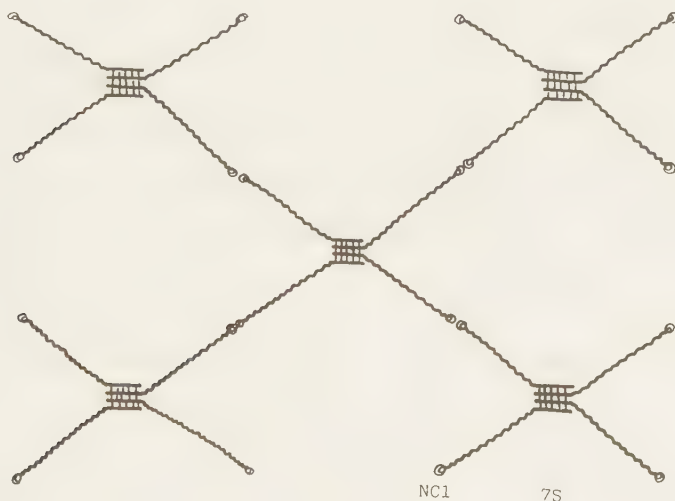


Figure 3. Model of the type IV collagen network.
Crosslinking occurs at the 7S and NC1 domains.

It has been reported that laminin can interact both with type IV collagen and with the heparan sulphate proteoglycan also present in basement membrane (84). This proteoglycan, however, appears to interact with laminin only and not with type IV collagen. It can be hypothesized, then, that the GBM is organized with type IV collagen in a middle layer interacting with laminin on both sides and by way of laminin with an outer layer of heparan sulphate proteoglycans.

On electron microscopy the type IV collagen can be seen localized to the lamina densa while heparan sulphate proteo-

glycans, laminin and possibly fibronectin are localized to the lamina rara (5). Thus the proteoglycan appears to be in the appropriate position to form the negative charge barrier, and laminin and fibronectin to be in a position to mediate the attachment of endothelial and epithelial cells to the GBM.

Glomerular basement membrane disease

A number of disease processes can result in an altered structure of the GBM (85). The most common causes are injuries induced by toxic agents, immunologic mechanisms, congenital and hereditary biochemical defects as well as injury where the mechanisms are unknown (Table IV). The following discussion will be restricted to immunologic mechanisms.

Table IV.

<u>Alterations of GBM in disease</u>	
<u>Kind of injury</u>	<u>Examples</u>
Toxic agents	aminonucleoside nephrosis aminoglycoside antibiotics
Immunologic mechanisms	deposition of immune complexes reaction of antibody with antigen in GBM
Congenital and hereditary defects	Alports syndrome Polycystic renal disease Congenital nephrotic syndrome
Other mechanisms	Diabetes mellitus Dense deposit diseasen

Immunologic mechanisms. In studies extending over some 70 years most forms of glomerulonephritis have been shown to be caused by either of two humoral immune mechanisms (86). Both involve deposition of antibodies within the kidney tissue. The antibodies may react directly with insoluble antigens (87), which can be either structural components of the basement membrane or antigens that are trapped in the kidney, i.e.

immunoglobulins or bacterial products. Alternatively antibodies may react with soluble non-glomerular antigens in the blood. The immune complexes formed will remain in circulation until they become trapped nonspecifically in the glomerular filter. Examples of such antigens include those of exogenous origin, like drugs and microbial antigens and those of endogenous origin, like nuclear proteins and tumor antigens.

Either mechanism will result in the accumulation in the kidney of immunoglobulins, which may give complement activation (88). Fragments of complement factors, namely C3a and C5a, are produced and function as chemotactic agents for leukocytes that accumulate where the antigen - antibody-complement are localized (89). Leukocytes release lysosomal proteases that may destroy the proteins of the basement membrane (90). Other mechanisms could involve monocytes (91) or platelets (92) and activated proteases in the coagulation system which may cause destruction of basement membrane components (93).

Experimental models of anti-basement membrane antibody disease. Immune responses directed against renal antigens has been reported as far back as in 1900 by Lindemann (94) who described that injection of heterologous anti-kidney antibody caused rabbits to develop proteinuria. This nephrotoxic serum model was later studied in more detail by Masugi (95) in the 1930's. The disease has since then often been referred to as Masugi nephritis. In the 1950's Krakower and Greenspoon (7) showed that GBM contains the major antigens reacting with the nephrotoxic antisera.

After injection of heterologous anti-GBM antibodies, two phases of the disease are observed (96). In the first

heterologous phase, the antibodies bind to GBM and, if sufficient amounts of the antibodies are bound, complement activation ensues. Within minutes leucocytes accumulate and fragmentation of GBM can be seen. The second, autologous phase (or delayed phase) occurs 7-10 days later when the host makes an immune response to the injected heterologous immunoglobulin, which acts as an antigen bound to the GBM. This phase accentuates the glomerular damage. Damage occurs most frequently in the glomerulus, probably as a result of rapid disappearance of antibodies from other organs due to poor fixation (96). The nature of the antigens involved is largely unknown, although it has been suggested that the antigen is a 50 000 dalton sialoprotein (97). Furthermore, it has been found that heterologous anti-type IV collagen and anti-laminin antibodies are nephritogenic in mice (98). There is no evidence for the presence of auto-immune antibodies in nephrotoxic nephritis.

Animals, however, can be stimulated to develop auto-immune anti-basement membrane antibodies. In 1962 Steblay (99) described an auto-immune nephritis induced in sheep immunized with human GBM. The sheep had linear deposits of immunoglobulin and complement along GBM as well as along the tubular basement membrane. Further support of the auto-immune nature of the disease was that it could be transferred to a normal sheep by transfusion of the circulating antibodies (100). The disease in sheep can be initiated by immunization with homologous GBM (101) as well as by immunization with GBM from several species including man, dog, rabbit and rat and interestingly also with human lung basement membrane (102). Although the sheep have glomerular and tubular lesions, lung hemorrhage as seen in Goodpasture's syndrome has not been observed in sheep even when immunized with human lung.

Anti-basement membrane antibody disease in man

In 1919 Goodpasture (103) drew the attention to the association of massive pulmonary hemorrhage with associated glomerulonephritis. The syndrome described occurred mostly in young men with rapidly progressive renal failure and crescentic glomerulonephritis without systemic disease. It has been shown to be caused by an antibody response against antigens present in the glomerular basement membranes (86).

The antibodies were first shown as a continuous linear deposit of immunoglobulin along GBM (Fig. 4) by Scheer and Grossman (104). The immunopathologic role of the anti-GBM antibodies was demonstrated by Lerner et al (105) who could transfer the disease to primates by either circulating antibodies or antibodies eluted from kidneys of patients. The results were later confirmed by observation of recurrence of anti-GBM antibody



Figure 4. Immunofluorescence analysis of a kidney biopsy from a patient with Goodpasture's syndrome. The kidney section was overlaid with FITC-labelled anti-human IgG. (By courtesy of Dr. Svend Larsen, Copenhagen)

induced glomerulonephritis in a renal transplant in a patient who had remaining circulating anti-GBM antibodies.

Later, antibodies directed against basement membrane components have also been associated with other disorders such as tubulointerstitial nephritis (106), bullous pemphigoid (107) and in disease involving intestinal basement membrane (87).

Antigens involved in anti-GBM nephritis. Work by Lindemann (94), Masugi (95) and Krakower and Greenspoon (7) have demonstrated that the GBM contains antigens reacting with specific antibodies in a manner causing glomerulonephritis. The antigens are linearly distributed along the inner layer of the GBM (108). Electron microscopy has shown that the antibodies are localized to the lamina densa (109) or to the lamina rara interna (110).

The classical example of antibody mediated glomerulonephritis is Goodpasture's syndrome, as discussed above. Till now however, the nature of the antigen has been unknown since presented data have been conflicting. Several lines of evidence accumulated during recent years (111,112,paper IV), however, support the idea that the Goodpasture antigen is noncollagenous in nature. Studies on the isolation and characterization of this antigen will be discussed in the chapters on paper IV and V.

Diagnosis of anti-GBM nephritis. Rapidly progressive glomerulonephritis with or without concomitant lung hemorrhage (i.e. Goodpasture's syndrome) is the typical clinical picture in anti-GBM glomerulonephritis. The diagnosis, however, should be based on immunofluorescence to detect antibodies bound in a linear pattern along the glomerular basement

membrane or by detection of specific circulating anti-GBM antibodies. Further verification can be obtained after elution of the antibodies from the kidneys by showing that they bind to GBM.

About 70% of patients with anti-GBM glomerulonephritis also have detectable antibodies directed against the tubular basement membrane (113). Some patients have antibodies fixed in all tubules while others have such fixed only in a few tubules (87). These variations in distribution has been assumed to represent differences in antibody specificity rather than limited accessibility of the antigen or different titers of antibodies. Antibodies also bind to antigens in the lung basement membrane, where they can be detected using direct immunofluorescence. Furthermore, the antibodies recognize antigens common to lung and glomerular basement membranes, since antibodies eluted from the lung can be used to stain GBM (87). Some cases clinically show only glomerulonephritis, with no involvement of the lung. It is not known whether these patients also have antibodies fixed to lung basement membranes. It has been suggested, however, that antibodies from patients with lung involvement have a wider specificity for basement membrane antigens than antibodies from patients with glomerulonephritis only (114).

Detection of circulating anti-GBM antibodies. Circulating anti-GBM antibodies were first detected using indirect immunofluorescence. Later more sensitive immunossays such as radioimmunoassay or ELISA have been used. These methods are listed in Table V, and more extensively discussed in the chapter on paper II.

Table V. Immunoassay for the detection of circulating anti-GBM antibodies

Reference	Technique	GBM antigen	Label	Antibody class assayed
Wilson et al (115) 1974	double antibody RIA	Collagenase digest	¹²⁵ I antigen	IgG
Mahieu et al (116) 1974	PEG precipitation	heat extracted glycoprotein fraction	¹²⁵ I antigen	IgG, IgM, IgA
McPhaul&Mullins (117) 1976	double antibody RIA	collagenase or trypsin digest	¹²⁵ I antigen	IgG
Lockwood et al (118) 1979	solid-phase RIA	collagenase digest	¹²⁵ I second antibody	IgG
Wilson (119) 1980	double antibody RIA	immunosorbent purified collagenase digest	¹²⁵ I antigen	IgG
Buffalo et al (120) 1980	double antibody RIA	8M urea extraction	¹²⁵ I antigen	IgG
Wieslander et al (11, 121) 1981	solid-phase ELISA	collagenase digest or 6M Gu-HCl extraction	alkaline phosphatase second antibody	IgG IgG, IgM, IgA
Wheeler&Sussman (122) 1981	solid-phase ELISA	collagenase digest	alkaline phosphatase second antibody	IgG
Papaioanno & Klassen (123) 1982	solid-phase fluorescence immun assay	collagenase digest	FITC labelled second antibody	IgG, IgM, IgA

Immunofluorescence is less sensitive than radioimmunoassay or ELISA (87) and has several drawbacks compared with these methods. Examples are the extensive specificity controls, essential since it has been shown that linear deposits of immunoglobulins along GBM can be found in apparently normal kidneys (124,125), in kidneys from patients with diabetes mellitus (126), and in kidneys from some cases of systemic lupus erythematosus (127). Immunofluorescence, however, has the advantage of screening for additional antibodies, for example such reacting with antigens in tubular basement membranes, brush border and cell nucleus (124) and can therefore be used as a complement to other assays.

PRESENT INVESTIGATION

An altered function of the GBM is an essential feature of glomerulonephritis. Therefore it is of clinical importance to understand its structure. Of particular interest is characterization of the antigenic properties since glomerulonephritis often involves an immune response i.e. interaction of antibody with specific antigens in the kidney. New alternatives in treatment of renal failure, such as plasmapheresis, have emphasized the need for precise clinical diagnosis. This has made better characterization of the glomerular antigens desirable, as they can be used for diagnostic purposes, selecting specific therapy and follow up.

The aim of this investigation has been to study antigenic components of the GBM in order to set up specific assays for anti-GBM antibodies and finally to isolate and characterize the antigens involved.

Antigens of GBM (paper I)

Proteins of GBM are highly crosslinked. Therefore disruptive methods have to be employed to solubilize its constituents. One way is to use proteases that selectively cleave the peptide backbones. Bacterial collagenase was used to selectively degrade the triple helical parts of the membrane, pepsin to degrade nontriple helical domains and trypsin to specifically cleave at lysine and arginine residues. Solubilization was also obtained with the less specific proteases pronase and papain.

Components solubilized from GBM using these enzymes were studied with regard to contents of various antigenic structures. Antigenicity was detected using antibodies that had been

raised in rabbits against particulate GBM. Since electro-immunoassay was used, only those molecules were detected that precipitated i.e. those containing more than one antigenic site. A further limitation was that molecules having neutral or positive net charge at the pH used (8.6), would not move towards the anode in the electric field and therefore escape detection.

The results expectedly showed that both pronase and papain efficiently solubilized the GBM but almost completely degraded the molecules leaving very little antigenic material. Trypsin or pepsin solubilized about half of the protein in the membrane. Collagenase solubilized 75% of the membrane and gave preparations with high antigenicity. Since pepsin digestion mainly releases basic triple helical molecules only few antigenic components should be expected when this enzyme was used. The digests obtained with collagenase, trypsin and pepsin all contained crossreacting antigens, probably representing the protease resistant regions for example of the 7S collagen and of laminin. Further studies of the antigens released by collagenase digestion, using gel chromatography on Sepharose 6B, demonstrated that at least seven antigens could be partially separated. All antigens were heterogenous in nature and each was probably present on several fragments since they showed size heterogeneity. Therefore most antigens eluted in mixtures. One high molecular weight antigen and another antigen of low molecular weight could, however, be isolated on the column.

Previous immunochemical studies have similarly shown that GBM contains a number of components having different antigenicity. One study indicated three components (59) while other studies

(111,128) presented data showing at least seven components with different antigenicity. Thus it has been shown that a number of components retaining antigenicity can be released from GBM by digestion with proteolytic enzymes. These studies provide important background for future work. It has not been clear whether the rabbit antiserum raised against particulate GBM contains antibodies with the same specificity as those found in serum of patients with nephritis. It has been shown, however, that the rabbit antiserum does not recognize the Goodpasture antigen (paper IV).

Assay for anti-GBM antibodies (paper II)

Several immunoassays for detecting anti-GBM antibodies have been described (see Table V). The radioimmunoassay developed by Wilson et al (115,119) is the most widely used. Several other radioimmunoassays have been described (116,117,118,120), and more recently ELISA has been introduced (paper II,121,122), as well as an assay using immunofluorescence (123).

We preferred ELISA techniques to radioimmunoassay since they allow rapid analysis of large numbers of samples using stable reagents. Furthermore there are no limitations caused by potential loss of antigenicity upon labelling with isotope. A main problem with ELISA and other solid phase assays is the difficulty to control coating of the antigen to plastic. Different antigens will coat with different efficiency, which could result in minimal coating of proteins under study. There is also the problem of release of antigens during assay, which will give rise to some of the variations seen in ELISA.

An ELISA assay can be divided in three steps: coating of antigen, incubation with serum and finally incubation with anti-

body-enzyme conjugate (Fig. 5).

The antigen preparation used for coating determines the specificity of the assay. A number of antigens have been used in assays for anti-GBM antibodies. Soluble antigens have usually been prepared by collagenase (115,117,118,119) or trypsin (117) digestion of GBM. Other procedures such as heat extraction (116), extraction with 8M Urea (120) or 6M guanidine-HCl (121) have also been used. Antigens solubilized by collagenase have been widely used because these antigens give an assay that is rather selective for antibodies from serum of patients with Goodpasture's syndrome, while sera from other subsets of glomerulonephritis show little reactivity (87,paper III).

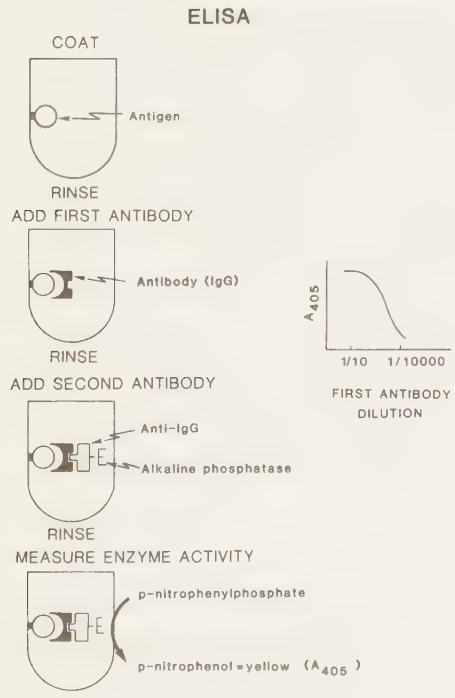


Figure 5. Principles of ELISA.

Initially we decided to coat the tubes using the two main components of the membrane, i.e. the globular domains solubilized by collagenase and the triple helical domains solubilized by pepsin. Antigenic components of the globular domains were adsorbed to the polystyrene tubes in almost any buffer. We have later shown, however, that dissociation of the antigen in 6M guanidine-HCl gives a much higher coating efficiency, possibly by dissociating aggregates of the molecules and thereby exposing a larger number of the antigenic determinants to the antibodies. It was subsequently shown that patients with Goodpasture's syndrome have antibodies only to the globular domains and not to the triple helical collagen.

The conditions in the third step, i.e. incubation with the antibody enzyme conjugate, largely determines the background. We found that the most crucial factor for decreasing the background was to include bovine serum albumin during incubation. The enzyme activity was measured by incubation with the fluorogenic substrate methylumbelliphenyl phosphate to obtain the highest sensitivity. In later studies, however, we have observed that the sensitivity using paranitrophenyl phosphate is quite satisfactory. Therefore we have subsequently avoided the time consuming measurements of fluorescence and preferred automated measurements of absorbance in a Multiscan photometer.

In later work we also increased the incubation temperature to room temperature, thereby allowing shorter incubations and a much faster assay, important in routine diagnostic work (121).

Antibodies in different forms of glomerulonephritis (paper III)

During the initial studies using the ELISA, we found that some patients had circulating IgG, IgA and IgM antibodies against the antigens solubilized by collagenase. These patients did not have the classical Goodpasture's syndrome but often had other types of systemic diseases like systemic lupus erythematosus. Thus it is possible that in various forms of glomerulonephritis there are antibodies of several immunoglobulin classes directed against different antigens of the GBM. If so, characterization of antibody and antigen should assist in the differential diagnosis of glomerulonephritis. Attempts were therefore made to selectively solubilize as many different types of antigens as possible by using a variety of extraction procedures. The two main domains of the membrane were solubilized by enzymes (collagenase or pepsin) and nonfragmented molecules by extraction with guanidine-HCl. Material insoluble after extraction with guanidine-HCl was solubilized by subsequent enzyme digestion and material insoluble after enzyme digestion was extracted with guanidine-HCl. Samples of the extracts were also reduced and alkylated in the hope of exposing further antigens. The solubilized antigens were coated to microtiter plates and sera from representative patients with different forms of glomerulonephritis were assayed for presence of reacting antibodies.

All patients with Goodpasture's syndrome had antibodies to antigens solubilized with collagenase. After reduction of the preparations the antibodies did not bind to the antigen, showing that intact disulphide bonds are required for antigenicity. Guanidine-HCl mainly released material which reacted with antibodies from patients with other types of

glomerulonephritis, e.g. IgA nephritis. The antigenicity of these preparations was not decreased by reduction. Thus, there are at least two types of antigens involved in antibasement membrane antibody nephritis, one specific for Goodpasture's syndrome and one related to other forms of systemic disease. Other types of antigens may be present but escape detection, since the solid phase assay used does not detect antibodies to antigens that do not coat or coat in such a way that the antigenic sites are masked.

These results corroborate work demonstrating antibasement membrane antibodies in nephritis other than Goodpasture's syndrome (116,129) and show that these antibodies are directed to antigens other than those active in Goodpasture's syndrome. There are, however, some overlaps of the reactivities. For example some patients with systemic lupus erythematosus show low but distinct reactivities with collagenase solubilized antigens. Subsequent work, however, has shown that a purified Goodpasture antigen does not react with those sera and only reacts with Goodpasture sera (130).

An interesting observation is that GBM prepared from sonicated glomeruli has a higher relative content of the Goodpasture antigen while GBM prepared by detergent procedures contains a higher relative proportion of the other types of antigens. This is the expected finding since patients with IgA nephritis have antibodies localized to the mesangial areas. The antibodies therefore appear to react with mesangial antigens enriched in GBM prepared by detergents.

Isolation of the antigen in Goodpasture's syndrome (paper (IV)).

There are conflicting data regarding the nature of the GBM antigen in Goodpasture's syndrome. One study (131) suggested that the antigen is collagenous since kidney sections digested with collagenase did not bind Goodpasture antibodies. Mahieu et al (132) similarly showed that the binding of Goodpasture antibodies to GBM antigens was inhibited by a peptide containing disaccharides isolated from GBM. They suggested that the antibodies were directed to the triple helical collagen domains of GBM. More recent studies (133) have indicated that the Goodpasture antibodies react both with the type IV collagen molecule and with laminin isolated from the EHS tumor. It was, however, not determined whether the antigenicity was destroyed by digestion with collagenase.

Marquardt et al (111) showed that GBM antigens solubilized by collagenase blocked the binding of Goodpasture antibodies to kidney sections and inhibited the binding of Goodpasture antibodies to GBM antigens in radioimmunoassay. The conclusion was that the antigen represents a noncollagenous glycoprotein. Molecules in the collagenase digest have been studied by polyacrylamide gel electrophoresis and two major components of 27 000 and 53 000 dalton were shown to react with Goodpasture antibodies (134).

Another study (135) using components isolated from the EHS sarcoma showed that neither laminin nor type IV collagen bound sera from Goodpasture patients, while a homogenate of the tumor bound such antibodies.

Further support of the noncollagenous nature of the Goodpasture antigen has been obtained by affinity chromatography of collagenase digests of basement membrane on a column

containing bound Goodpasture antibodies (112). None of the components bound had an amino acid composition typical for collagen.

From the results of paper II and III we concluded that solubilization of the GBM with collagenase is a useful step in the isolation of the Goodpasture antigen. The ELISA was modified to a competitive assay for the Goodpasture antigen which could be used to determine contents of the antigen in fractions during purification. The antibody used was from the serum of a Goodpasture patient.

The Goodpasture antigen was isolated by chromatography of collagenase digests of GBM on Sephacryl S-200 eluted with an associative buffer, followed by affinity chromatography and finally by chromatography on Sephacryl S-200 in a dissociative buffer. The smallest molecule carrying the antigen was a polypeptide with a molecular weight of 26 000 dalton as determined by polyacrylamide gel electrophoresis. Its amino acid composition showed low contents of glycine and hydroxyproline indicating that it was not triple helical collagen. The protein was also present in aggregates, probably dimers, that had the same amino acid composition and contained the same antigenic site as the 26 000 dalton protein. A proportion of the larger molecules could be reduced to form the 26 000 dalton protein. After reduction, however, reactivity of all antigens with Goodpasture antibodies were lost, showing that the antigen contained disulphide bridges. All seven Goodpasture patients studied, had circulating antibodies to the isolated 26 000 dalton protein as well as to the larger forms of the antigen. It appears then that only one antigen is involved in the pathogenesis of Goodpasture's syndrome.

Origin and nature of the Goodpasture antigen (paper V)

Already the size and amino acid composition of the isolated Goodpasture antigen monomer (the 26 000 dalton molecule), indicated that it may represent a collagenase resistant globular domain of type IV collagen. Further experiments were aimed at verifying this hypothesis.

Native bovine type IV collagen contains an antigen reacting with the Goodpasture antibodies, since binding of such antibodies to human Goodpasture antigen could be inhibited by this collagen. Furthermore isolated human Goodpasture antigen could inhibit the binding of Goodpasture antibodies to a collagenase digest of the bovine type IV collagen. Immunoblotting of a collagenase digest of the bovine type IV collagen showed that Goodpasture antibodies stained proteins which migrated to the same position as the reactive proteins in a collagenase digest of human GBM i.e. Goodpasture antigen. The non-digested type IV collagen did not enter the gel and showed no component in the gel staining with the antibodies. Therefore release of the antigens occurs upon cleavage of the triple helix with collagenase, i.e. the antigen is located on resistant portions of the collagen. To obtain sufficient quantity of the type IV collagen to allow chemical characterization of the fragment containing the Goodpasture antigen, it was necessary to use type IV collagen prepared by reduction. Unfortunately this reduction destroys the antigenicity. A collagenase resistant fragment, which migrated to the same position as an antigenic fragment isolated from bovine GBM, could be isolated. These two fragments had very similar amino acid composition. The results further support the localization of the Goodpasture antigen to collagenase resistant fragments of the type IV collagen.

The size of the fragment and its amino acid composition indicates that it may represent the NC1 peptide. Further proof is the finding, using antibodies from the same patient, that the NC1 peptide isolated from human placenta basement membrane reacts with these antibodies and crossreacts with the purified Goodpasture antigen described in paper IV (R. Timpl, personal communication).

We conclude that the Goodpasture antigen is derived from the collagenase resistant globular domain of type IV collagen. All seven Goodpasture patients had antibodies against the antigens liberated by collagenase digestion of the type IV collagen. All basement membranes contain type IV collagen, but only GBM and sometimes lung basement membrane are affected in Goodpasture's syndrome. The antigen can, however, be isolated from lung, placenta and kidney basement membranes. The yield is highest from GBM and lowest from placenta (unpublished results). This could indicate that the organs affected most are those with largest exposure to circulating antibodies and the highest concentration of Goodpasture antigen and therefore have the potential of binding larger amounts of antibody.

SUMMARY

The aim of the present study was to identify antigens involved in antibody mediated nephritis, in particular Goodpasture's syndrome. One goal was to isolate basement membrane antigens specific for subgroups of nephritis with circulating anti-basement membrane antibodies, which could be useful in the differential diagnosis of nephritis. Sensitive assays for soluble antigens and for circulating antibodies were needed both for the purification of antigens and identification of patients with antibasement membrane nephritis.

Initial studies were aimed at solubilizing antigens from GBM using digestion with proteolytic enzymes. Subsequently other procedures including extraction with chaotropic agents were tried. Indeed some specificity for different antigens was shown, as antibodies from patients with Goodpasture's syndrome were found to react most strongly with collagenase digests of basement membrane, while antibodies from other patients with nephritis reacted mainly with antigens extracted with guanidine-HCl. The Goodpasture antigen was purified from collagenase digests of GBM. The antigenic site(s) was located on a polypeptide with a molecular weight of 26 000. The structural data on this purified antigen indicated similarity with a peptide from type IV collagen. It was subsequently shown that the collagenase resistant globular domain of type IV collagen contained the antigenic site.

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APPENDIX: PAPERS 1-V.

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HUMAN GLOMERULAR BASEMENT MEMBRANE HETEROGENEITY OF ANTIGENIC DETERMINANTS

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Summary

Human glomerular basement membrane was solubilized by digestion with proteolytic enzymes and immunoreactive components were quantitated and characterized by using rabbit antibodies raised against the particulate membrane. A number of antigens were demonstrated but they did not separate on gel filtration. However, two antigenic components in a collagenase digest of the membrane could be separated and isolated by Sepharose 6B chromatography. Chemical characterization suggests that both fragments are noncollagenous glycopeptides (molecular weights approx. 1 000 000 and 60 000–200 000, respectively).

Introduction

Contradictory data on the structure and immunoreactivity of basement membranes have been published [1–5]. Consequently, the biosynthesis of basement membranes is presently only partly understood [6–13]. Some of the reasons for the variability are variations in the primary structure of the collagenous and glycoprotein components respectively, the extent and nature of molecular interactions and the alterations brought about by degeneration, regeneration and by ageing. An important problem is to obtain pure basement membrane components for structural studies and for the study of alterations and immunological specificities in certain diseases, notably diabetes and glomerulonephritis [3,14,15].

The purpose of the present study was to investigate if it was possible to prepare pure immunoreactive basement membrane fragments for characterization and use in studies of the pathophysiology of nephritis.

Methods and Materials

Preparation of human glomerular basement membrane and antibodies

Human glomerular basement membrane was prepared according to the method of Spiro [16] as modified by Westberg [14]. Lyophilized human glomerular basement membrane (10 mg/ml) was suspended in 1 M NaCl, mixed with an equal volume of Freund's Complete Adjuvant and injected subcutaneously in the neck of rabbits (1–5 mg doses). Booster doses in Freund's Incomplete Adjuvant were given several times at 4-week intervals. The rabbits were bled 12 days after the last injection and sera were pooled and checked for antibody titer by using electroimmunoassay. The antisera were adsorbed with insolubilised plasma as described by Avrameas and Ternynck [17]. Adsorbed sera reacted immunologically with the soluble fraction of collagenase digested human glomerular basement membrane but did not react with human plasma nor with red cell membrane, platelet or cartilage extracts. Therefore it appears that neither cell structures nor nonspecific connective tissue components react with the antisera. Gamma-globulins were purified according to Steinbuch and Audran [18] and again tested for specificity for human glomerular basement membrane by using immunoelectrophoresis, Ouchterlony double diffusion and crossed immunoelectrophoresis as discussed below.

Immunological methods

Immunoelectrophoresis was performed as described by Grabar and Williams [19] and double diffusion in Agarose gel according to Ouchterlony [20]. Electroimmunoassay according to Laurell [21] was performed in 1% Agarose gel containing 1–2 ml/25 ml of purified antihuman glomerular basement membrane gamma-globulin. Fused rocket immunoelectrophoresis was performed according to Svendsen [22] and crossed immunoelectrophoresis according to Clarke and Freeman [21]. Immunoelectromigration was done according to Grubb [24]. Essentially the same buffer (0.025 M barbital, pH 8.6) was used in all procedures. The plates were washed with saline, dried and stained with Coomassie brilliant blue.

Chemical methods

Hydroxyproline was determined as described by Stegeman and Stadler [25] after hydrolysis of the glomerular basement membrane for 18 h in 6 M HCl at 105°C in sealed tubes. Total phosphorus was measured after digestion of the basement membrane preparation in 70% perchloric acid using the ascorbic acid method of Chen et al. [26].

Total protein was measured as described by Lowry et al. [27] but the protein concentration in chromatographic fractions was usually monitored by their absorbance at 280 nm.

The amino acid composition of glomerular basement membrane and subfractions was determined on a Durrum amino acid analyzer, after the sample had been hydrolyzed for 24 h at 110°C in 6 M HCl under argon. 3- and 4-hydroxyproline were separated on a Biocal amino acid analyzer. The same colour factor of 4-hydroxyproline was used for calculating the amounts of these two amino acids. Contents of neutral sugars were determined by gas chromatography of alditol acetates essentially as described by Axelsson and Heinegard [28].

TABLE I

PROTOCOL FOR FRAGMENTATION OF BASEMENT MEMBRANE PREPARATIONS WITH PROTEOLYTIC ENZYMES

Enzyme	Amount of enzyme (mg)	Membrane (mg)	Incubation time (h)	Temp. (°C)	Buffer
Collagenase (CLSPA Worthington)	(0.37 + 0.18 + 0.09)	75	72	37	5 ml 0.001 M HEPES/0.01 M CaCl ₂ , pH 7.5
Pepsin (Sigma)	(0.5 + 0.5)	50	48	21	5 ml 0.01 M acetic acid
Trypsin (Type III, Sigma)	(1)	53	9.5	37	5 ml 0.1 M Tris-HCl, pH 8.0
Pronase (Merck AG)	(1)	31	72	37	5 ml 0.15 M Tris-HCl/0.01 M CaCl ₂ , pH 7.4
Papain (2 × crystallized, Sigma)	(0.1)	10	4	65	2 ml 0.05 M sodium phosphate/0.005 M cysteine-HCl/0.005 M EDTA, pH 6.5

Proteolytic fragmentation of human glomerular basement membrane

Enzymic digestion of basement membrane was performed using the conditions shown in Table I.

All digests were centrifuged at $40\,000 \times g$ for 1 h at 4°C. Residual pellets were dialyzed against several changes of redistilled water, and were lyophilized. Supernatants were tested for immunoreactive components by using electro-immunoassay with collagenase digested membrane as a standard. Rocket height was shown to be proportional to the amounts of membrane in concentration ranges from 200 µg/ml to 2 mg/ml.

Gel chromatography

Solubilized basement membrane samples were applied to a 1.8×180 cm Sepharose 6B column, which had been equilibrated with 0.15 M NaCl/0.05 M Tris-HCl buffer, pH 7.4. Elution was carried out with the same buffer and 8-ml fractions were collected. The effluent fractions were monitored for protein content by their absorbance at 280 nm and tested for contents of antigen by the electroimmunoassay procedure described above. Fractions with identical antigenic activity were pooled (as indicated in Fig. 1) and concentrated by ultrafiltration (Amicon type UM-2 membrane).

Results and Discussion

Purity of the human glomerular basement membrane preparations

The purity of the preparations was studied by using both light and electron microscopy. All preparations appeared to be free of undisrupted glomerulae and cells. The average hydroxyproline content was 7.2%. The total phosphate content was less than 0.1% indicating no, or insignificant, contamination by phospholipids, RNA or DNA. The amino acid, neutral sugar and hexosamine composition of the human glomerular basement membrane preparation is pre-

sented in Table II. The amino acid composition with high relative contents of glycine, proline, hydroxyproline and hydroxylysine are similar to data previously published by others [1,14,31]. The hydrolysate also contained a component which eluted early on the amino acid analyzer (before hydroxyproline) and which was present in a hydrolysate of isolated basement collagen but not in a hydrolysate of guinea pig skin type I collagen. In separate experiments it was shown that a corresponding peak was a major component in hydrolysates of Telomycin (a gift from Bristol Laboratories, U.S.A.) which is known to contain 3-hydroxyproline [29]. It is therefore likely that this component is 3-hydroproline, which is unique for basement membrane collagen. The relative carbohydrate composition is also well correlated to data in the literature [1,14] with approximately equimolar amounts of glucose and galactose, which is typical for basement membrane. The content of mannose, fucose, glucosamine and sialic acid is related to oligosaccharides in the non-collagenous pro-

TABLE II

CHEMICAL COMPOSITION OF INTACT AND FRAGMENTED HUMAN GLOMERULAR BASEMENT MEMBRANE (HGBM)

Amino acids as residues per 1000. Carbohydrates as weight percent. Values in parentheses are weight ratios to glucose.

	HGBM	Sephacose 6B fractions of collagenase digested HGBM			
		I	IIa	IIb	III
3-Hydro	28.9	n.d.	n.d.	n.d.	n.d.
4-Hydro	98.0	trace	trace	trace	trace
Asp	64.6	89.8	86.2	88.0	91.0
Thr	32.8	49.4	54.8	64.7	60.3
Ser	45.5	77.4	107.1	99.4	102.6
Glu	91.5	127.0	119.0	108.8	118.2
Pro	70.2	61.3	66.2	71.2	65.8
Gly	221.1	189.8	197.7	132.2	149.8
Ala	50.6	78.5	66.7	79.8	76.0
Cys	18.3				
Val	35.2	49.7	48.8	51.9	63.6
Met	9.2				
Ile	31.1	29.5	30.9	40.0	40.2
Leu	62.2	77.5	66.5	78.8	73.1
Tyr	15.2	13.2	15.5	26.8	16.8
Phe	27.0	25.4	28.9	39.2	26.4
Hylys	26.5	20.5	21.0	11.1	trace
Lys	18.2	32.8	24.7	33.2	43.0
His	13.8	21.4	21.0	25.9	27.4
Arg	40.1	56.8	45.2	48.9	45.9
Total hexose	8.2	1.08	1.20	0.74	0.91
Glucose	3.5 (1)	0.36 (0.14)	0.43 (1)	0.24 (1)	0.32 (1)
Galactose	3.8 (1.09)	0.41 (1.14)	0.45 (1.05)	0.33 (1.38)	0.31 (0.97)
Mannose	0.73 (0.21)	0.22 (0.61)	0.21 (0.49)	0.09 (0.37)	0.13 (0.41)
Fucose	0.20 (0.06)	0.05 (0.14)	0.05 (0.12)	0.027 (0.11)	0.075 (0.23)
Glucosamine	1.02 (0.29)	0.6 (1.67)	n.d.	n.d.	0.19 (0.59)
Galactosamine	0.14 (0.04)	0.14 (0.39)	n.d.	n.d.	0.22 (0.69)
Sialic acid	0.64 (0.18)	0.23 (0.64)	0.14 (0.33)	0.20 (0.83)	0.28 (0.88)

n.d., not detected.

teinaceous part of the basement membrane. The analytical data indicate that the basement membrane preparation was pure, with little or no contamination by other proteins, Table II.

Collagenase digestion of human glomerular basement membrane

Approximately 75% of the human glomerular basement membrane and almost all of the collagenous components, as indicated by hydroxyproline, was solubilized by digestion with bacterial collagenase (Table III). The digest was fractionated on Sepharose 6B and the elution profile is shown in Fig. 1. Practically all of the hydroxyproline-containing material was eluted in the most retarded peak from the column (peak IV). The fractions were tested for antigenic activity by fused rocket immunoelectrophoresis, shown in Fig. 1. Two antigens were separated from the bulk of the others, indicated by I and III in Fig. 1. The separation of two fragments with different antigenicity was confirmed by using immunoelectromigration (Fig. 2), double radial immunodiffusion (not shown) and crossed immunoelectrophoresis (Fig. 3). The intermediate fractions (indicated by 'II' in Fig. 1) contained several different antigens. Some of them were shared by several fractions, and also by the material of small size (III in Fig. 1). In conclusion, two antigens in collagenase digested basement membrane can be separated from the others by gel chromatography on a Sepharose 6B column. In addition several other antigens are present but elute in a mixture. It was shown separately that neither of the two components I and III contained detectable amounts of plasma proteins. From calibration chromatograms with plasma proteins it was estimated that the molecular sizes of components I and III (Fig. 1) were in the order of 1 000 000 and less than 200 000, respectively.

The amino acid, neutral sugar, and hexosamine contents of the fractions indicated in Fig. 1 are presented in Table II. Antigenic components (I, II and III) prepared by Sepharose 6B chromatography of collagenase digested base-

TABLE III

ENZYME DIGESTION OF HUMAN GLOMERULAR BASEMENT MEMBRANE

	Collagen- ase	Pepsin	Pronase	Trypsin
OH-proline in supernatant (g/l)				
Protein in supernatant (g/l)	0.073	0.036	0.063	0.039
OH-proline in pellet (mg)				
Dry weight of pellet (mg)	0.005	0.099	0.043	0.083
OH-proline in supernatant (mg)				
OH-proline in supernatant + pellet (mg)	0.982	0.220	0.931	0.280
Percentage HGBM solubilized	77	44	91	51
Immunological identity (+) or nonidentity (—) of digests				
Collagenase vs.		+	—	+
Pepsin vs.	+		—	+
Pronase vs.	—	—		—
Trypsin vs.	+	+	—	

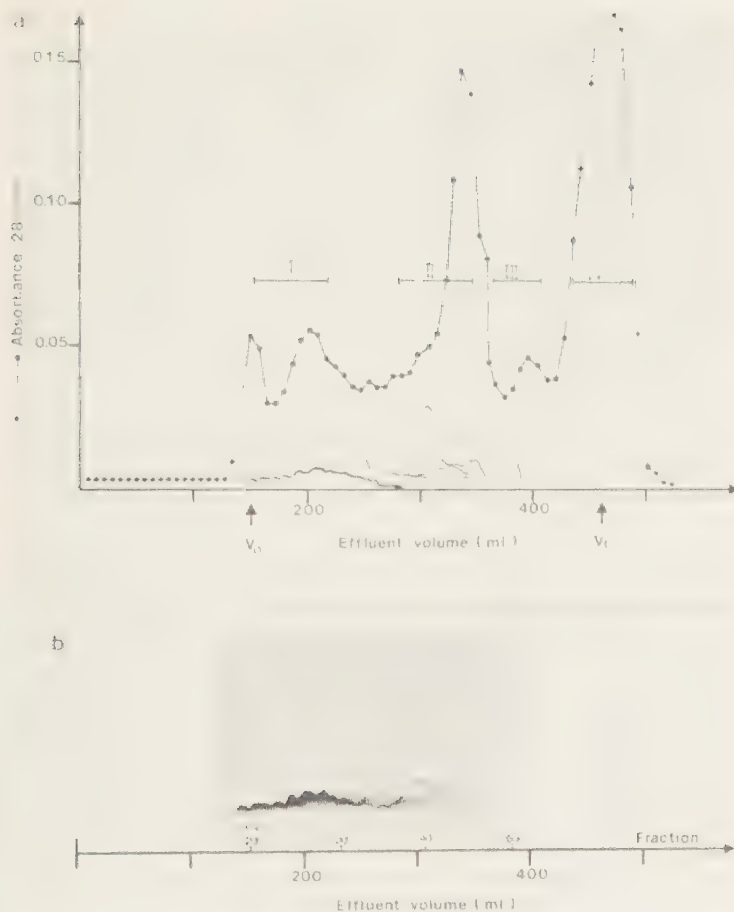


Fig. 1. Analysis of components solubilized by collagenase digestion of human glomerular basement membrane. a. Sepharose 6B elution profile. Insert: schematic representation of fused rocket analysis of fractions (shown in b). b. Fused rocket analysis of Sepharose 6B fractions displaying the interrelation between antigens in different fractions. Continuous precipitation lines are formed for each antigen-antibody system.

ment membrane contained only trace amounts of hydroxyproline and the glycine was significantly lower than in the glomerular basement membrane preparation. Neither cysteine nor methionine was found and the hydroxylysine content was low, especially in those antigenic components most retarded in their elution. Therefore, it is likely that these antigens are not of collagenous nature. In support, the carbohydrate composition of the Sepharose 6B fractions indicate lower content of the collagenous part of the basement membrane with lower content of glucose and galactose and higher content of the other carbohydrate residues.

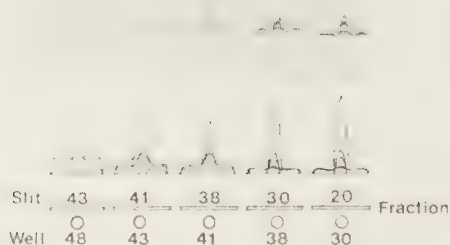


Fig. 2. Immunological identities of Sepharose 6B fractions of collagenase digested human glomerular basement membrane tested by immunoelectromigration analysis.

Pepsin, trypsin, pronase and papain digestion of human glomerular basement membrane

Both pepsin and trypsin digestion of basement membrane solubilized several antigenic components as indicated by the number of rockets seen on analysis of fractions from Sepharose 6B chromatograms (Figs. 4 and 5). Unfortunately, the concentration of antigens in most fractions was too low to give visible precipitation lines for interpretation of continuity in fused rocket analysis or crossed immunoelectrophoresis. These experiments were not repeated since it could be shown that antigenic fragments released by pepsin and trypsin digestion were immunologically identical to antigens in corresponding fractions from Sepharose 6B chromatograms of collagenase digested basement membrane (summarized in Table III). Prolonged digestion with pepsin or trypsin did not result in destruction of antigenic sites (data not shown). In contrast, upon

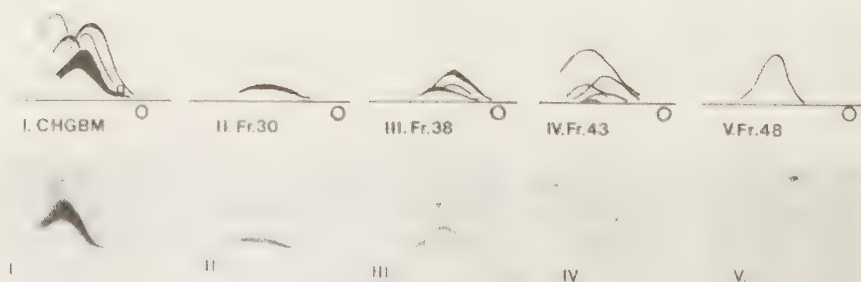


Fig. 3. Crossed immunoelectrophoresis according to Clarke and Freeman of Sepharose 6B fractions of collagenase digested human glomerular basement membrane (as indicated in the figure) against anti-human glomerular basement membrane gammaglobulin.

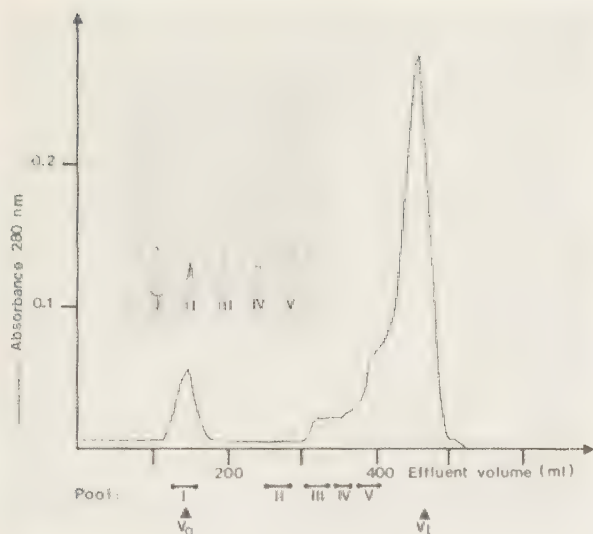


Fig. 4. Sepharose 6B chromatogram of components solubilized by pepsin digestion of human glomerular basement membrane. Insert: electroimmunoassay of pooled fractions. Bars indicate pooled fractions.

pronase digestion, antigens were first released and then successively degraded until at 72 h only one antigen could be identified (Fig. 6). None of the antigens solubilized upon digestion with pronase crossreacted with antigens in collagenase, pepsin or trypsin digests (Table III). Incubation with papain efficiently solubilized the basement membrane but no antigenic activity remained after 4 h of incubation. As with collagenase, digestion with pronase released both the

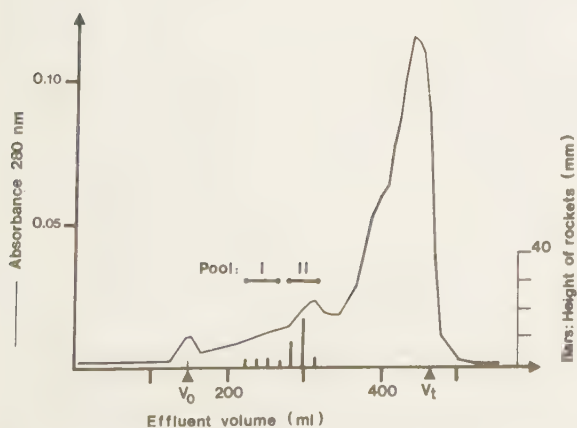


Fig. 5. Sepharose 6B chromatogram of components solubilized by trypsin digestion of human glomerular basement membrane. Vertical bars indicate the rocket height in electroimmunoassay of the fractions.

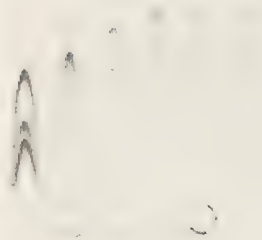


Fig. 6. Components solubilized from human glomerular basement membrane by pronase digestion. Electroimmunoassay of samples at: (from left to right) less than 1 h, 1, 2, 3, 4, 5, 7, 24 and 72 h of digestion.

collagenous and the noncollagenous component of the basement membrane while trypsin digestion primarily released noncollagenous components. Pepsin digestion gave intermediate results (Table III).

General Discussion

In recent years numerous investigators have shown that the chemical structure and the molecular organisation of glomerular basement membrane is very complex [30–33]. The extensive crosslinking of the components by covalent disulphide bonds and the collagen type of crosslinks as well as other less known interactions render the membrane quite insoluble. Therefore, procedures in which such bonds, or more often peptide bonds, are cleaved have to be used, and a wide variety of heterogenous fragments have been solubilized. An additional factor for increasing the complexity may be limited *in vivo* proteolysis of the proteins [3,30]. Only few basement membrane subunits have been isolated in a form homogenous enough to make chemical characterization possible and the yields have been low (Ohno et al. [32] and Kefalides [33]).

When this investigation was started it was presumed that some of the difficulties encountered in chemical analysis might be resolved by using immunochemical methods. Since the first report by Krakower and Grenspon [34] on the nephritogenic property of dog glomerular basement, the interest in basement membrane immunology has been centered around the problem of autoimmunisation against membrane components in glomerulonephritis (for review, see Refs. 36 and 5). Usually antigens have been solubilized by means of enzymic digestion, and in a few instances by mechanical or chemical extraction [5,35,36]. Kefalides [37] has suggested that there are three antigenic sites in basement membranes in general. Two sites are considered to be located in a procollagen-like molecule: one in the triple helical portion and the other in the

non-helical extension of the molecule. A third site is supposed to be located in a large molecular weight matrix glycoprotein.

This interpretation is probably an oversimplification, since the total number of antigens detected rises when resolution is enhanced by using for instance crossed immunoelectrophoresis [38].

From analyses of the amino acid and sugar contents of fractions from the Sepharose 6B chromatogram of basement membrane preparations which had been digested with various proteases, it appears that fractions containing antigenic fragments did not contain any collagenous components. It is likely then, that the antigenic sites demonstrated are mainly located in the non-collagenous, non-triple-helical portions of the basement membrane.

It is of interest to note that it was possible to cleave the membrane in such a way that fragments containing single antigenic sites could be identified. However, the large number of molecules within each antigenic class makes isolation by methods based on size or electrical charge difficult. It was hoped as an alternative that some of the various solubilization procedures used would liberate more homologous antigens into solution, but this was not the case with any of the methods tested. Enzymatic cleavage by several enzymes did not yield fragments with fewer antigenic sites. On the other hand, the quantitative electro-immunoassay and the fused rocket technique made it possible to study the enrichment of the antigens in different fractions and, as discussed above, two antigenic fragments isolated by Sepharose 6B chromatography of collagenase digested membrane could be separated from the bulk of the others. The two fractions isolated are still chemically heterogeneous or polydisperse. The smaller molecular weight material, most retarded on Sepharose 6B, seems to be immunochemically homogeneous but chemically heterogeneous or polydisperse. This antigen crossreacts immunologically with an antigen isolated from normal human urine, while the determinant located in the large fragment is never demonstrated in urine [39], whether normal or pathological. Further information on the composition of human glomerular basement membrane may be gained from studies of degradation products in the urine.

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Anti-basement membrane antibody: immunoenzymatic assay and specificity of antibodies

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A sensitive enzyme linked immunosorbent assay has been developed for circulating anti-glomerular basement membrane antibodies and optimal conditions for each step have been determined. Structurally different preparations of antigens from glomerular basement membrane are coated to the walls of polystyrene tubes. Sera containing either specific antibody or negative control are added and allowed to react. Immunoglobulins bound to the antigens on the tube are then detected using anti-immunoglobulin antibodies coupled to alkaline phosphatase. The enzymic activity is measured using the fluorogenic substrate methylumbelliphenyl phosphate. The fluorescence recorded is proportional to the concentration of specific antibodies in the serum tested. The specificity of the assay has been determined. The two different structural components of glomerular basement membrane were used as antigens. Type IV collagen was prepared after pepsin digestion, while glycoprotein components were solubilized by digestion of glomerular basement membrane with collagenase. Sera from rabbits immunized with purified type IV collagen reacted with type IV collagen only, while serum from a rabbit immunized with a glycoprotein component of human glomerular basement membrane reacted with collagenase digests of glomerular basement membrane only. Sera from several patients with antibody mediated nephritis (Goodpasture's syndrome) reacted with glycoprotein components in collagenase digests, but not with type IV collagen prepared from human glomerular basement membrane.

Key-words: glomerular basement membrane; glomerulonephritis; Goodpasture's syndrome; immunoassay

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Antibodies directed against antigenic determinants in or associated to basement membrane

are considered to induce the disease in certain forms of glomerulonephritis [16, 24]. Although the clinical expression of the disease may vary [2, 3, 10, 31], the majority of the patients present

with a rapidly progressive glomerulonephritis often in combination with lung haemorrhage, Goodpasture's syndrome [31]. A number of patients do not survive and many of the surviving patients have end-stage renal failure and require dialysis treatment or kidney transplantation. Recent improvements in the therapy of the disease involve use of plasma exchange to remove circulating antibodies specific for basement membrane [18]. The prognosis is much improved when therapy is started early, i.e. depends on early diagnosis.

Until recently the diagnosis of anti-basement membrane antibody nephritis was confirmed by demonstration of a smooth, linear deposit of immunoglobulins along the glomerular basement membrane of renal biopsy specimens [23, 27, 32]. These immunoglobulins represent specific antibodies, which are adsorbed to the target organ. [16]. The titre of circulating antibodies in the blood are usually very low. In some patients, however, circulating antibodies directed against basement membrane antigens can be demonstrated using comparatively insensitive indirect immunofluorescence [32]. More sensitive procedures allow the detection of such circulating antibodies in almost all cases of Goodpasture's syndrome [3]. Therefore demonstration of circulating antibodies directed against basement membrane will assist early diagnosis. Furthermore presence of such antibodies in patients with renal failure presents an indication to delay kidney transplantation to avoid recurrence of disease in the transplant. The specificity of the antibodies has not been established. The glomerular basement membrane contains two distinct structural components, type IV collagen and glycoproteins. Conflicting data regarding antibody specificity for either of these components have been published [19, 21, 31].

In this report an enzyme linked immunosorbent assay (ELISA) for anti-basement membrane antibody is presented. It can be used in studies of the antibody response in experimental nephritis [4]; to quantitate antibodies in sera from patients with anti-basement membrane nephritis; to monitor for effects of therapy and in studies aiming at characterisation of the antigens involved in the disease of these patients in order to improve differential diagnosis.

MATERIALS AND METHODS

Preparation of antigens. Human glomerular basement membrane (HGBM) was prepared essentially according to Westberg [28] as described previously [29]. Basement membrane glycoproteins (CHGBM) were solubilized by digestion of glomerular basement membrane with 1/50 (w/w) of collagenase for 72 h in 0.05 mol/l HEPES buffer, 0.01 mol/l CaCl_2 , pH 7.5. The collagenase used (CLS Worthington) had been purified according to Lee-Own & Andersson [14]. Nonspecific proteolytic activity could not be detected in this preparation using ^3H -acetylated haemoglobin as the substrate. Human glomerular basement membrane type IV collagen was prepared as described elsewhere [30], essentially according to Kresina *et al.* [11] and Sage *et al.* [22].

Chemical methods. Amino acid analysis was performed using a Durrum amino acid analyser after hydrolysis of collagenase digested human glomerular basement membrane and type IV collagen in 6 mol/l HCl at 110°C for 24 h under argon. Sodium dodecyl sulphatepolyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% gels was done according to Laemmli [12] as described by Weber & Osborn [26]. Samples (50 µg) were applied to 5 × 60 mm gels. Reference proteins were purchased from Bio Rad Laboratories, Calif., USA.

Immunological methods. Rabbits were immunized with the following antigens:

1. Human IgG (Kabi AB, Stockholm, Sweden) (100 µg) purified by gel chromatography on Sephacryl S-200 (Pharmacia Fine Chemicals, Uppsala, Sweden), two rabbits.
2. Human glomerular basement membrane (2 mg), two rabbits.
3. Human glomerular basement membrane type IV collagen (0.5 mg), one rabbit.
4. A low molecular weight glycoprotein fraction (Fraction III, 0.5 mg) prepared by Sepharose 6 B (Pharmacia Fine Chemicals, Uppsala, Sweden) chromatography of a collagenase digest of glomerular basement membrane [29], one rabbit.

All antigens were suspended in Freund's complete adjuvant mixed with an equal volume of 0.15 mol/l NaCl and injected s.c. in the neck of separate rabbits. Booster doses with the same

amounts of antigen in Freund's incomplete adjuvant were given after 4 weeks and then every second week until antibody titres were constant.

Immunoglobulins were purified from rabbit antisera as described by Steinbuch & Audran [25]. Rabbit antihuman IgG to be used for conjugation with alkaline phosphatase was purified by affinity chromatography on an immunosorbent column of human IgG coupled to Sepharose CL 4B according to March [20]. Antibodies were eluted with 0.2 mol/l glycine-HCl buffer, pH 2.8. Covalent coupling of rabbit anti-human IgG with alkaline phosphatase (Sigma type VII, Sigma, St. Louis, Mo., USA) was done with glutaraldehyde according to Avrameas [1]. In some instances commercially available (Orion Diagnostica, Helsinki, Finland) alkaline phosphatase conjugates of swine anti-human IgG or swine anti-rabbit IgG were used.

Unbound IgG and free enzyme were removed from all immunoglobulin-enzyme conjugates used in ELISA by chromatography on Sephacryl S-200 eluted with 0.15 mol/l NaCl, 0.05 mol/l phosphate buffer, pH 7.5 also containing 0.05% sodium azide and 1% bovine serum albumin (BSA). The conjugates recovered in the void volume peak were stored at 4°C in the buffer used for the gel chromatography.

Enzyme linked immunosorbent assay (ELISA) was done essentially according to Engvall & Perlmann [5] as follows: disposable polystyrene tubes were coated with 200 µl of the indicated amount of antigen dissolved in 0.05 mol/l sodium carbonate buffer, pH 9.6, or in the case of collagen 0.1 mol/l acetic acid. The tubes were incubated at room temperature (21°C) for 16 h and washed three times. This and subsequent rinses were done with 0.15 mol/l NaCl, 0.05% (v/v) Tween 20. The tubes were drip-dried upside down for a few min. Sera to be analysed were diluted with incubation buffer (0.15 mol/l NaCl, 0.05 mol/l phosphate buffer, 0.05% (v/v) Tween 20, pH 7.5) and 200 µl of the diluted serum was added to each tube. After incubation at 4°C for 6 h the tubes were washed three times with 0.15 mol/l NaCl, 0.05% (v/v) Tween 20. Conjugate, 200 µl of a suitable dilution (determined by a chessboard titration) in incubation buffer supplemented with 2 g/l BSA, was added to each tube. After incubation for 16 h at 4°C the tubes were washed three times. Substrate solution containing 0.05 mol/l

of 2-amino-2-methyl-1,3-propanediol, pH 9.6 with 5 mol/l MgCl₂ and 2 µmol/l of 4-methylumbelliphenyl phosphate (Koch Light Laboratories, England) was added. The reaction was stopped after incubation for 30 min at 37°C by addition of 1 ml of 0.05 mol/l Na₂CO₃ [6, 13]. Fluorescence was measured using an Aminco Bowman Spectrofluorometer, excitation wavelength 364 nm and emission wavelength 448 nm.

Reproducible fluorescence values were obtained by using a standard solution of 1 mg/l quinine sulphate (Sigma, St. Louis, Mo., USA) in 0.1 mol/l of sulphuric acid for calibration. The instrument was set to a fluorescence value of 50 with the meter multiplier set at 0.03 at wavelengths of 350 nm for excitation and 450 nm for emission.

RESULTS

Antigens

Glomerular basement membrane contains at least two distinct antigenic components: one is glycoprotein in nature and the other type IV collagen, which is unique for basement membranes [8, 9]. The amino acid compositions of the soluble portion of the collagenase digest of human glomerular basement membrane and the purified type IV collagen are summarized in Table I. The glycoprotein component has been

TABLE I. Amino acids analysis (residues/1000) of glomerular basement membrane solubilized by collagenase digestion (CHGBM) and human type IV collagen (H IV coll).

	CHGBM	H IV coll
3-hydro	n.d.	12.1
4-hydro	20.4	109.8
Asp	86.5	54.9
Thr	53.1	25.8
Ser	71.3	43.2
Glu	132.8	85.9
Pro	60.8	64.9
Gly	140.7	306.5
Ala	69.6	40.3
Cys	28.9	n.d.
Val	41.3	25.6
Met	6.4	8.8
Ile	33.6	33.7
Leu	74.0	53.6
Tyr	25.5	4.9
Phe	30.6	24.1
His	22.6	7.0
Hylys	19.6	62.6
Lys	30.4	6.5
Arg	51.9	29.8

n.d. = not detected.

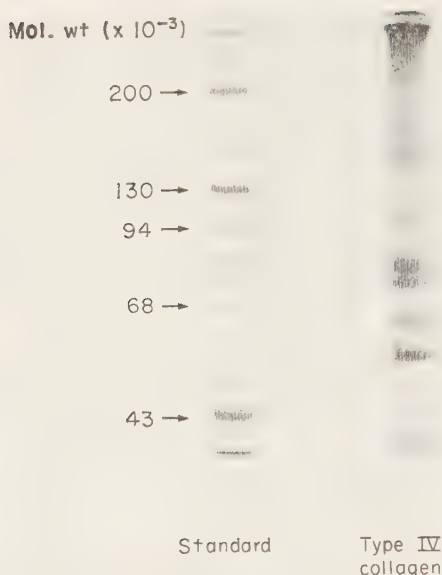


FIG. 1. SDS-PAGE (7.5% gels) of reduced type IV collagen prepared from human glomerular basement membrane.

described in some detail before and is not further discussed [29]. The high content of glycine, hydroxyproline and hydroxylysine and the low content of tyrosine indicate the purity of the type IV collagen preparation. It was characterized by electrophoresis on 7.5% SDS-polyacrylamide gels (Fig. 1). Components with apparent mol. wts of 140,000–160,000 and of about 85,000–50,000, respectively, were identified. The collagen components were completely degraded by digestion with purified bacterial collagenase as previously shown by SDS-PAGE [30].

Glomerular basement membrane preparations and collagenase digests thereof contain immunoglobulins [28, 29]. When such preparations are coated to polystyrene tubes, these immunoglobulins in addition to specific antibodies will react with the anti-immunoglobulin reagent, increasing the background in the ELISA. Therefore the effects of contaminating immunoglobulins on the specificity and sensitivity of the ELISA were investigated. In an initial experiment immunoglobulins present in the antigen preparation were removed using affinity chromatography. A collagenase digest of glomerular basement membrane was chromato-

graphed on a column containing rabbit anti-human serum antibodies coupled to Sepharose 4B by the cyanogen bromide procedure [20]. The material not bound to the column, containing basement membrane glycoproteins free from plasma proteins, was used to coat polystyrene tubes. When such purified antigens were coated the background fluorescence values in ELISA were reduced by 25%. It was found, however, that the background could also be reduced to negligible levels if BSA (2 g/l) was added to the incubation buffer containing anti-immunoglobulin-enzyme conjugate as described below. Removal of immunoglobulin contaminants from antigens is, therefore, not necessary.

Coating with antigens

Antigens were coated on the walls of polystyrene tubes, 11 × 55 mm in 200 µl of buffer. Optimal conditions for coating were tested by varying concentration, buffer ionic strength and pH, incubation time and temperature (see below). Glycoprotein antigen concentrations in samples used for coating were determined from analysis of the dry weights of the original basement membrane and the insoluble material remaining after digestion with collagenase, assuming that the difference provides an approximate estimate of the amounts of material solubilized in each preparation.

Glycoprotein antigens. The following buffers were tested:

- 0.05 mol/l sodium phosphate buffer, pH 7.5
- 0.05 mol/l sodium carbonate buffer, pH 9.6 with or without 1 mol/l of NaCl
- 0.05 mol/l sodium acetate buffer, pH 5.5

The values obtained in ELISA of one positive serum were identical independent of the buffer used, indicating that the coating efficiency was similar. Similarly increasing the ionic strength to 1 mol/l of sodium chloride in the carbonate buffer did not result in altered ELISA values. Coating with antigen for 16 h at 4 and 21°C was tested using the sodium carbonate buffer. Values obtained in ELISA, when expressed as the ratio between a positive serum and a control serum, were 1.9 for coating at 4°C and 2.6 for coating at 21°C. Antigen concentrations was important only when very low concentrations were used for

coating. The ELISA values remained linear even with concentrations of collagenase digest as low as 10 mg/l, provided that other factors were kept constant. For economy of antigen a concentration of 10 mg/l was therefore used in the final procedure. Coating was routinely performed in 0.05 mol/l sodium carbonate buffer, pH 9.6, overnight (16 h) at room temperature (21°C).

Type IV collagen. Collagen is soluble at low pH or in strong salt solutions. Therefore, two alternative solvents were used for coating: 0.1 mol/l acetic acid or 1 mol/l sodium chloride, 0.05 mol/l Tris HCl, pH 7.4. Coating in acetic acid gave lower background fluorescence values compared with 1 mol/l sodium chloride and was preferred. Incubation overnight (16 h) at room temperature (21°C) gave higher fluorescence values compared with incubation for 6 h at room temperature (21°C) or 16 h at 4°C. Antigen concentrations as low as 1 mg/l gave satisfactory coating. Routinely, coating was done in 0.1 mol/l acetic acid, overnight at room temperature (21°C).

Incubation with antisera

Optimal conditions for incubation with antiserum were tested using sera from rabbits immunized with either of human glomerular basement membrane, glycoprotein Fraction III [29] or type IV collagen. In addition, sera from 10 patients with anti-basement membrane nephritis were tested in the assay. The disease had been verified by direct and indirect immunofluorescence. Negative controls were pre-immune rabbit sera and sera from 25 healthy blood donors representing a wide spectrum of blood groups. The blood was allowed to coagulate at room temperature and serum was isolated by centrifugation. The serum was stored frozen until analysis. In a control experiment it was shown that the serum samples could be kept at room temperature for 1–2 weeks with minimal change of antibody titre.

The most important factor to reduce the non-specific binding of serum proteins (immunoglobulins) is presence of detergent (Tween 20, 0.05%) in the buffer and in the rinse solutions [5]. The time- and temperature-dependence were

tested using a specific antiserum (patient with Goodpasture's syndrome) diluted in 0.05 mol/l sodium phosphate, 0.15 mol/l sodium chloride containing 0.05% (v/v) Tween 20. The fluorescence recorded at 4°C increased from a value of 21 at 1 h to a constant level of 42, which was reached after 5 h. Incubation at 4°C gave lower background values than incubation at 21°C. An important consideration is the optimal dilution of the antiserum. Immunoglobulins in non-diluted sera may increase background and reduce sensitivity by non-specific binding to the walls of the tube. On the other hand, too extensive dilutions tend to reduce sensitivity. It is not practical to make serial dilutions of each serum to be tested. Positive human sera, however, when tested at a dilution of 1/20 gave high fluorescence values in the ELISA, while background as determined with blood donors serum remained low (Fig. 2). Rabbits immunized with glomerular basement membrane respond with very high titres and their sera could be diluted 1000-fold. Optimal conditions for incubation of serum was 6 h at 4°C in 0.15 mol/l NaCl, 0.05 mol/l phosphate buffer pH 7.4 containing 0.05% (v/v) Tween 20.

Incubation with conjugate

As compared to the results obtained with shorter incubation times, prolonged incubation with antibody-enzyme conjugate resulted in higher fluorescence values for at least 16 h. Therefore depending on the sensitivity required

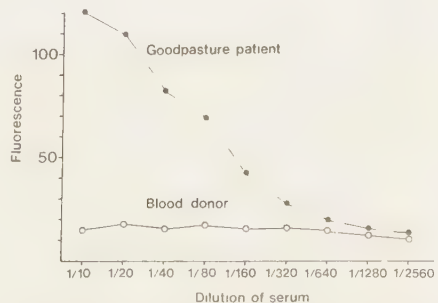


FIG. 2. ELISA of dilutions of a serum which contains anti-basement membrane antibodies. The tubes were coated with collagenase solubilized human glomerular basement membrane.

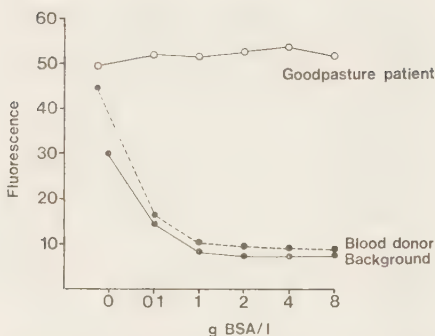


FIG. 3. Reduction of background and negative control fluorescence values in ELISA by inclusion of bovine serum albumin (BSA) during incubation with anti-IgG-antibody-enzyme conjugate.

the incubation may be varied. The background fluorescence was reduced to 50% by incubating at 4°C. When rabbit serum or BSA was included in the incubation buffer the fluorescence values of negative controls and background were considerably reduced while positive sera gave the same high fluorescence as without additions (Fig. 3). Therefore BSA (2 g/l) was used in all incubations with conjugate. The non-specific background could be further decreased by purifying the conjugate by gel chromatography on Sephacryl S-200 as discussed in Materials and Methods. Optimal conditions for incubation of conjugate was found to be 16 h at 4°C in a buffer of 0.15 mol/l NaCl, 0.05 mol/l phosphate, pH 7.4, containing 0.05% (v/v) Tween 20 and 2 g/l of BSA.

Specificity and sensitivity of ELISA

The specificity of the ELISA was tested. Sera from rabbits immunized with human type IV collagen reacted strongly in assays using tubes coated with type IV collagen, but not when antigens solubilized by collagenase digestion of basement membrane had been coated (Fig. 4). Sera from rabbits immunized with particulate basement membrane (anti-GBM) reacted with high fluorescence values with both collagen and glycoprotein antigens. The anti-fraction III antisera reacted only with basement membrane solubilized with collagenase (Fig. 4). This indicates that the type IV collagen preparation and the glycoprotein antigens solubilized by

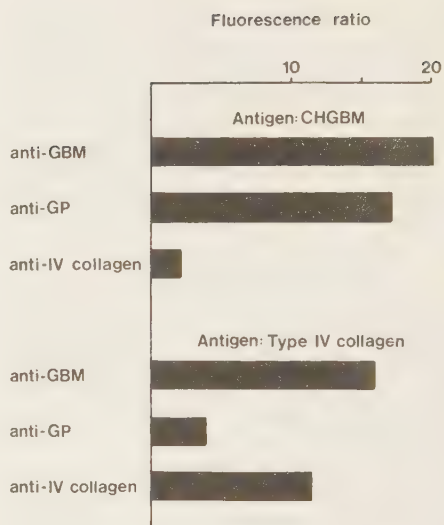


FIG. 4. Human glomerular basement membrane solubilized by collagenase digestion (CHGBM) and type IV collagen prepared from human glomerular basement membrane, respectively, were coated on the walls of polystyrene tubes. Antisera from rabbits immunized with different antigens were tested in ELISA: anti-GBM = serum from rabbit immunized with particulate human glomerular basement membrane; anti-GP = serum from rabbit immunized with a small molecular weight basement membrane glycoprotein, fraction III; anti-type IV collagen = rabbit immunized with type IV collagen prepared from human glomerular basement membrane. The bars represent the ratio between the fluorescence values found in ELISA for immune and preimmune sera of the respective rabbits.

collagenase digestion represent two antigenically different parts of the membrane. The diagnostic specificity of the ELISA was confirmed by demonstrating antibodies in sera from patients with suspected anti-GBM-disease. Sera from these patients reacted with collagenase solubilized antigens and gave high fluorescence values. In contrast the assay for anti-type IV collagen antibodies was negative (Fig. 5). All patients had kidney biopsies which showed linear deposits of antibody along the glomerular basement membrane. Furthermore circulating antibody could also be demonstrated using indirect immunofluorescence with sections of normal human kidney. In contrast sera from 25 healthy blood donors gave fluorescence values well below the range of all the positive sera (Fig. 5).

In order to define the detection limit of

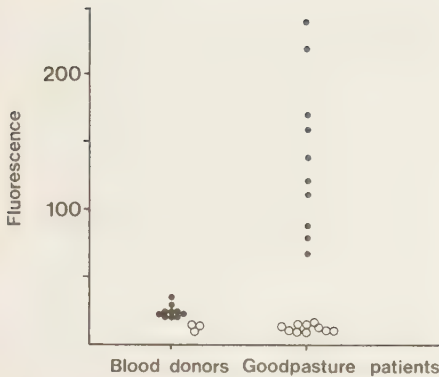


FIG. 5. Antibody specificity in patients with Goodpasture's syndrome ($n = 10$). Tubes were coated with glomerular basement membrane solubilized by digestion with collagenase (●) and type IV collagen (○). No patients showed reactivity with the type IV collagen preparation. Negative controls were sera from healthy blood donors ($n = 10$). Only three of the control sera were analysed for content of antibodies to collagen.

anti-HGBM-antibodies using ELISA one positive serum was serially diluted and gave positive values at a maximal 1000-fold dilution (Fig. 2). Other experiments using IgG coated tubes showed that antibody concentrations of less than $1 \mu\text{g/l}$ can be detected. The ELISA was applied in monitoring for decreased plasma antibody titre after plasma exchange. A patient with verified Goodpasture's syndrome, was treated at several occasions with plasma exchange. The antibody titre decreased after each treatment (Fig. 6).

Reproducibility

One major concern in a solid phase test such as the ELISA described here is the adsorption of the antigens to the polystyrene tube. Variability of coating efficiency may vary considerably from

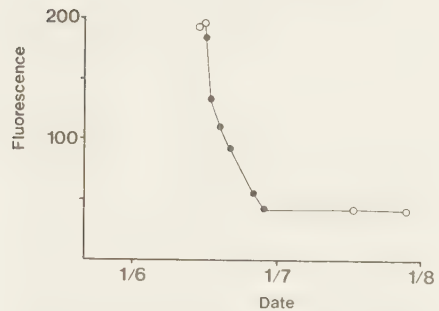


FIG. 6. Monitoring anti-GBM antibody titre in a patient treated with plasma exchange. Human glomerular basement membrane solubilized by collagenase digestion was used as the antigen in ELISA. Analysis of serum (○), and analysis of plasma from the first approximately 400 ml sample of each treatment (●).

one tube to another. Since antigens are non-covalently bound to the walls of the tube, leakage of antigens [15] may be considerable during the many steps of the assay. Strict standardization of antigens, materials and equipment will reduce the variability. The reproducibility of the ELISA was therefore tested by analyses of serum samples from one normal control person and one patient with Goodpasture's syndrome on three consecutive days. Eleven aliquots of each serum sample were analysed in parallel (Table II). Sera from 25 healthy blood donors were analysed in triplicate on two occasions. The sera were stored frozen during the 7 days in between analyses (Table II). Day to day variation was low and freezing and thawing had insignificant effects on ELISA results. Nevertheless it is advisable to include a standard negative control serum and a known positive serum control in each set of ELISA. Because of the variability in adsorption properties of tubes, it is important to analyse all samples in triplicate. To limit the pipetting error

TABLE II. Reproducibility of the ELISA. One serum sample from a blood donor and one from a patient with Goodpasture's syndrome were divided into 11 aliquots, which were each analysed on three consecutive days. Sera from 25 blood donors were analysed in triplicate on two occasions. Values are given as means $\pm$ SD

	1st	2nd	3rd analysis
One blood donor ($n=11$)	11 ± 0.6	9 ± 0.8	10 ± 0.8
One patient with Goodpasture's syndrome ($n=11$)	45 ± 4.2	51 ± 5.4	46 ± 3.6
Blood donors ($n=25$)	21 ± 4.5	18 ± 3.9	—

we use an automatic dispensing equipment with an error of less than 0.2%.

DISCUSSION

Patients with renal disease having anti-basement membrane antibodies may show a spectrum of glomerular, tubulointerstitial and lung affections [2, 3, 10, 31]. Several laboratories now use indirect immunofluorescence techniques to detect circulating antibodies against basement membrane antigens. The sensitivity is not as high as in ELISA or RIA and the immunofluorescence is at best semiquantitative. Recently, sensitive radioimmunoassay procedures have been partially described and applied to clinical problems [17, 19, 33]. Although their sensitivity is comparable with ELISA the latter is more convenient as reagents may be stored for extended periods and no sophisticated equipment is required.

HGBM is rather resistant to solubilization and procedures where disulphide, or more often, peptide bonds are cleaved, have to be used. Digestion of HGBM with collagenase solubilizes a very heterogeneous mixture of protein components which are also antigenically heterogeneous [29]. It is likely that this complex mixture contains only a low proportion of antigenic components, which can react with patient anti-GBM antibodies. Therefore, to obtain sufficient coating of specific antigen, the optimal concentration of CHGBM used for coating has to be relatively high.

The higher efficiency of coating at a higher temperature indicates a dependence on hydrophobic interactions. Increased salt concentration which would promote hydrophobic interactions, however, did not effect coating efficiency. It is possible that other interactions are of importance for coating or the higher salt concentration might affect the properties of the antigen. Adsorption and desorption of antigens vary between tubes resulting in a variable coating [15]. This factor is probably the major reason for the variability in ELISA. Therefore the use of tubes from the same batch in a set of analyses is essential for minimized variability.

Inclusion of detergent (Tween 20) in the first antibody step in ELISA (incubation of patient and control sera) is essential to avoid non-specific binding of immunoglobulins to the walls

of the tubes, directly or to the protein coat. Addition of protein (serum albumin, rabbit serum) to the diluted patient and control sera did not affect the reactivity in ELISA. In contrast addition of protein (bovine serum albumin or swine serum) in the second antibody step, with the enzyme-conjugate, considerably reduced background levels in ELISA. This reduction is probably due to elimination of non-specific, low-affinity binding of the conjugate to the tubes either directly or via other proteins.

A fluorogenic substrate in ELISA was preferred because of the 10 times higher sensitivity as compared with non-fluorogenic substrates.

The precision and repeatability of the ELISA is acceptable for routine use. It is advisable, however, to always include a positive control. A higher precision may be obtained by kinetic measurements of enzymatic activity, but compared with the variations due to coating, the added precision is marginal.

The immunologic and diagnostic value of the ELISA when applied for detection and quantitation of anti-GBM antibodies in patients is indicated by the correlation of strongly positive patient sera to anti-GBM antibody disease diagnosed by clinical and immunohistopathologic criteria. Positive sera could be diluted 1000-fold and still react in ELISA. Further confirmation of the immunologic specificity of the ELISA has been obtained in experimental autoimmune anti-GBM antibody disease in sheep [4].

One application of the test is to assist in obtaining early diagnosis of patients with rapidly progressive glomerulonephritis [17, 18]. Titres of circulating antibodies can be followed and in case of renal failure transplantation should be delayed until anti-GBM antibodies are not detectable, because of the risk of recurrence of disease in the transplant. Another application for ELISA is in the search for nephritogenic agents and antigenic sites in HGBM. Using ELISA it was shown that sera from patients with Goodpasture's syndrome did not react with HGBM components solubilized by pepsin digestion or with type IV collagen purified from pepsin digests of HGBM [30]. On the other hand when the collagen of the HGBM was selectively digested with collagenase, the solubilized proteins reacted in the ELISA. It

appears then, that the anti-GBM antibodies in Goodpasture's syndrome are directed against non-collagenous components. As described, the ELISA is completed in 40 h. The times have been chosen for optimal performance and can easily be reduced when few samples are to be analysed in clinical situations where early diagnosis is essential. Provided tubes have been coated in advance sample analysis can be completed in 6 h. Incubations are done at room temperature for only 2 and 4 h for the first and second step, respectively. The reduction of sensitivity is of little importance when assaying serum from patients with suspected Goodpasture's syndrome who usually have high titres of anti-GBM antibodies at presentation.

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Antiglomerular basement membrane antibody: Antibody specificity in different forms of glomerulonephritis

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Antiglomerular basement membrane antibody: Antibody specificity in different forms of glomerulonephritis. Components were solubilized from human glomerular basement membrane by digestion with collagenase and pepsin or by extraction with guanidine-HCl either directly or after previous digestion with the enzyme. The diverse preparations were used as antigens in the enzyme-linked immunosorbent assay (ELISA) of antibody titers in sera from patients with Goodpasture syndrome and patients with other forms of glomerulonephritis, that is, systemic lupus erythematosus, periarteritis nodosa, and IgA-related nephropathy. Patients with Goodpasture syndrome had high titers of IgG antibodies reacting most strongly with collagenase digests. The antigen(s) was only partly solubilized by guanidine-HCl extraction, was destroyed by pepsin digestion as well as reduction, and partly destroyed by trypsin digestion. The antigen(s) is most likely noncollagenous protein. Antibodies from patients with other forms of nephritis were directed primarily against antigen(s) in guanidine-HCl extracts, while the antigen(s) was not solubilized by collagenase digestion. Pepsin digestion destroyed the antigen(s). The antibodies were of a different class, that is, the patients with systemic lupus erythematosus had IgG and IgA as well as IgM antibodies; the patients with periarteritis nodosa had IgM or IgG and IgA antibodies, while the patients with IgA-related nephritis had the highest recorded titers of IgA but also had IgG as well as IgM antibodies. None of the patients had antibodies directed against triple helical collagen. The antibody response in anti-GBM antibody-related nephritis, then, is different both with respect to antigen and antibody class and depends on the underlying disease syndrome.

Anticorps anti-membrane basale glomérulaire: Spécificité des anticorps dans différentes formes de glomérulonephrites. Les constituants de membranes basales glomérulaires humaines ont été solubilisés par digestion avec de la collagénase et de la pepsine ou par extraction avec de la guanidine-HCl directement ou après digestion préalable par l'enzyme. Ces diverses préparations ont été utilisées comme antigène dans la titration d'anticorps par essai immunosorbant reliés par enzyme (ELISA) dans des sérums de malades ayant un syndrome de Goodpasture, et de malades avec d'autres formes de glomérulonephrites, notamment lupus érythémateux disséminé, périartérite noueuse, et néphropathie à IgA. Les malades ayant un syndrome de Goodpasture avaient des titres élevés d'anticorps IgG réagissant très fortement avec des produits de digestion de collagénase. L'antigène(s) était seulement partiellement solubilisé par extraction à la guanidine-HCl, était détruit par la digestion par la pepsine et par réduction, et était partiellement détruit par digestion à la trypsine. L'antigène(s) est probablement une protéine noncollagène. Les anticorps provenant de malades ayant d'autres formes de néphrites étaient essentiellement dirigés contre des antigènes provenant d'extraits guanidine-HCl, tandis que l'antigène(s) n'était pas solubilisé par digestion à la collagénase. La digestion par la pepsine a détruit l'antigène(s). Les anticorps étaient de différentes classes, les malades ayant un lupus érythémateux disséminé avaient des IgG, des IgA, et des IgM comme anticorps; les malades ayant une périartérite noueuse avaient des anticorps IgM, IgG, IgA, alors que les malades ayant une néphrite à IgA avaient les titres les plus élevés d'anticorps IgA, mais avaient également des IgG et des IgM. Aucun des malades n'avait d'anticorps dirigés contre du collagène à triple hélice

La réponse anticorps dans la néphrite liée à un anticorps anti-GBM est donc différente par la classe d'antigènes ou d'anticorps en fonction du syndrome sous-jacent.

Available data show that anti-glomerular basement membrane antibodies (anti-GBM antibodies) have a role in the pathogenesis of Goodpasture syndrome (GP) [1]. Kidney biopsy specimens from such patients show a linear nephritis on immunofluorescence microscopy [2]. In most cases circulating anti-GBM antibodies can be demonstrated using either indirect immunofluorescence microscopy [2], radioimmunoassay (RIA) [3-5] or enzyme-linked immunosorbent assay (ELISA) [6]. The character of the antigen or antigens reacting with anti-GBM antibodies have not been determined. Collagen as well as noncollagen glycoproteins of the glomerular basement membrane (GBM) have been proposed to contain the antigenic sites responsible for immunization [4, 7]. More recently, some patients, who do not fulfill the clinical criteria of Goodpasture syndrome have been shown to have circulating anti-GBM antibodies [8]. Studies in our laboratory, using ELISA, have confirmed this finding and indicated that the specificity as well as the immunoglobulin class of the anti-GBM antibodies may vary with the underlying disease [9, 10]. Therefore, the character of antigens used in assays of anti-GBM antibodies is important.

This study was designed to compare the efficiency of several GBM preparations to show the specificity of anti-GBM antibodies in sera from a number of patients representing several defined clinical syndromes

Methods

ELISA. IgG, IgA, and IgM antibodies reacting with isolated human glomerular basement membrane antigens were measured using ELISA, as described elsewhere [6, 10]. In essence, antigens (200 μ l, 10 μ g/ml) dissolved in 0.05 mole/liter sodium carbonate, pH 9.6, containing 0.05% sodium azide were coated on wells in polystyrene microtiter plates (Nunc Immunoplate, Roskilde, Denmark) by incubation at 22°C for 16 hr. To obtain optimal coating with type IV collagen, however, this antigen was solubilized in 0.5 mole/liter acetic acid as previously described [11]. The wells were rinsed threefold in 0.15 mole/l

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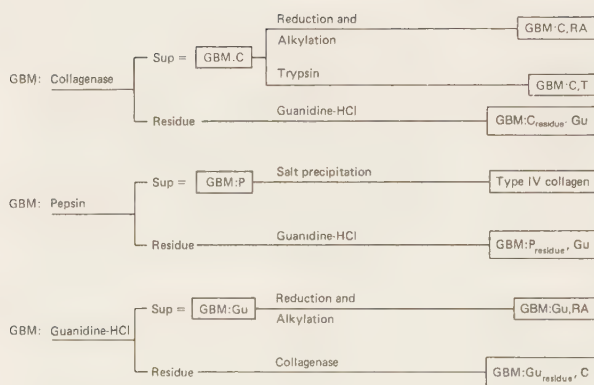


Fig. 1. Preparation of antigenic components from GBM: Flow diagram. For details, see Methods.

liter sodium chloride, 0.05% (v/v) Tween 20. Dilutions (1/20, 200 μ l) of patient or control sera in 0.05 mole/liter sodium phosphate buffer pH 7.4, 0.15 mole/liter sodium chloride, were added and incubated at 4°C for 6 hr. The wells were rinsed as above and suitable dilutions [200 μ l in 0.15 mole/liter sodium chloride, 0.05 mole/liter sodium phosphate buffer, pH 7.4, 0.05% (v/v) Tween 20, 2 g/liter of bovine serum albumin] of swine antihuman IgG, IgA, or IgM conjugated to alkaline phosphatase (conjugates from Orion Diagnostica, Helsinki, Finland) were added. After incubations for 16 hr at 4°C, the plates were rinsed as above and enzyme substrate (200 μ l/well), p-nitro-phenylphosphate (Sigma Chemical Corp., St. Louis, Missouri) in 1 mole/liter diethanolamine buffer, pH 9.8 containing 5 mmoles/liter magnesium chloride, was added. The colored reaction product obtained after incubation (22°C, 1 hr) is proportional to the content of specific antibody in the sera tested. A Titertec Multiscan spectrophotometer (Flow Laboratories, McLean, Virginia) equipped with a 405-nm filter was used for absorbance measurements. The specificity of the conjugates were tested and no crossreactivity between Ig-class (IgG, IgA, and IgM) could be demonstrated using ELISA.

Preparation of antigens from GBM. The different antigens used in this study were prepared from GBM as summarized in the flow diagram, Figure 1. Details are given below.

Preparation of human glomerular basement membrane. Human cadaver kidneys were obtained at autopsy within 24 hr after death. Glomerular basement membrane was prepared essentially as described by Westberg and Michael [12]. The following protease inhibitors were included in all steps of the preparations: Benzamide-HCl 5 mmoles/liter, 6-aminocaproic acid 25 mmoles/liter, EDTA 10 mmoles/liter, phenylmethanesulfonylfluoride 1 mmole/liter, N-ethylmaleimide, 5 mmoles/liter, served a dual function as a protease inhibitor and to prevent disulphide exchange.

Collagenase digestion. Bacterial collagenase (Type CLS, Worthington, Freehold, New Jersey), purified according to Lee-Owen and Anderson [13], was used to digest GBM at 37°C for 72 hr [6, 10]. Lyophilized GBM was suspended (5 mg/ml) in

0.05 mole/liter Hepes buffer, pH 7.45, containing 0.01 mole/liter calcium chloride, and all protease inhibitors but EDTA, at concentrations described above. A residual pellet after guanidine-HCl extraction of GBM was dialyzed extensively against the buffer and then digested. After digestion preparations were centrifuged at $\times 100,000g$ for 1 hr, and the supernatants were used as antigens in ELISA.

Trypsin digestion. GBM was solubilized by collagenase digestion, extensively dialyzed against 0.1 mole/liter sodium phosphate buffer, pH 7.6, and digested with diphenylcarbamyl chloride-treated trypsin (Sigma) at a ratio of trypsin to substrate of 1:50. Digestion was carried out at 37°C for 4 hr. The digest was clarified by centrifugation at $\times 100,000g$ for 1 hr, and the supernatant was used as an antigen in ELISA.

Pepsin digestion and isolation of type IV collagen. GBM was suspended in 0.5 mole/liter acetic acid and digested with pepsin (Sigma) at 4°C for 16 hr at a ratio of enzyme to dry weight GBM of 1:50. The digest was centrifuged at $\times 100,000g$ for 1 hr, and the supernatant was directly used as an antigen or for the preparation of type IV collagen as described previously [6, 11].

Guanidine-HCl extraction of GBM antigens. Lyophilized GBM was extracted at 3 mg/ml and 42°C for 18 hr with 6 moles/liter guanidine-HCl, 0.05 mole/liter Tris-HCl, pH 7.5, also containing the protease inhibitors listed above for the preparation of GBM. Residual pellets after collagenase digestion and pepsin digestion, respectively, were similarly extracted with guanidine-HCl. The extracts were clarified by centrifugation at $\times 100,000g$ for 1 hr before use in ELISA.

Reduction and alkylation of antigens. Selected antigens, indicated in Figure 1, were reduced, alkylated, and tested for immunoreactivity in ELISA. Solubilized components were dialyzed extensively against 6 moles/liter guanidine-HCl, 0.05 mole/liter Tris-HCl, pH 7.5. Reduction of these components was performed at 37°C for 5 hr with less than 0.5 mg of protein per ml of 5 mmoles/liter dithiothreitol. After 5 hr iodoacetic acid (threefold excess) was added, and alkylation was carried out overnight at room temperature.

Precipitation with ethanol of extracts and digests. Macromol-

Table 1. Contents of collagen (hydroxyproline) and total protein (α -amino nitrogen) in antigenic components prepared as described in Figure 1

	Hydroxyproline α -Amino nitrogen	
	Molar ratio $\times 100$	Arbitrary units
GBM	5.2	1.00
GBM:C	1.8	0.34
GBM:C:residue, Gu	5.3	1.03
GBM:P	5.3	1.04
Type IV collagen	6.4	1.23
GBM:P:residue, Gu	4.8	0.92
GBM:Gu	3.3	0.63
GBM:Gu:residue, C	2.7	0.52

ecules solubilized by enzyme digestion or by extraction with guanidine-HCl were precipitated with 10 volumes of 95% ethanol at 4°C and left overnight. The precipitates were recovered by centrifugation at $\times 2000g$ for 30 min at 22°C.

Quantitation of collagen (hydroxyproline) and total protein (α -amino nitrogen). The hydroxyproline contents of GBM and antigens precipitated with ethanol were determined colorimetrically according to Stegemann and Stalder [14] after hydrolysis of samples in 6 moles/liter HCl for 24 hr at 104°C. Protein (α -amino nitrogen) contents of GBM or antigens precipitated with ethanol were determined by the ninhydrin procedure [15] after hydrolysis of samples in 6 moles/liter HCl for 24 hr at 104°C [15].

Electrophoresis. Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis was done essentially according to Laemmli [16] as previously described [10, 11]. Samples to be electrophoresed were precipitated with 10 volumes of ethanol and redissolved in sample gel buffer containing 2% SDS with or without 1% mercapto-ethanol.

Patients. Thirty-two patients were included in the present study. Eighteen patients represented classical Goodpasture syndrome having lung purpura, rapidly progressive glomerulonephritis, and crescentic glomerulonephritis on light microscopy. Serum samples from these patients were obtained from several nephrology centers in Sweden and Denmark. In all patients diagnosis had been confirmed by the finding of a linear pattern upon immunofluorescence microscopy. Circulating anti-GBM antibodies were also demonstrated by indirect immunofluorescence microscopy using normal kidney sections. Patients with other types of nephritis and having antibodies to basement membrane were obtained from an ongoing study on glomerulonephritis at the Department of Nephrology, University Hospital, Lund (to be published). Seven patients had systemic lupus erythematosus with multiorgan involvement, a positive ANF titer, and low levels of complement. Two patients had multiorgan vasculitis and glomerulonephritis. They were diagnosed as having periarthritis nodosa. The immunofluorescence pattern was granular in the nine non-Goodpasture patients with systemic connective tissue disease, indicative of immunocomplex-related glomerulonephritis [2]. Five patients had IgA-related glomerulonephritis, focal glomerulonephritis on light microscopy, and mesangially localized IgA and C'3 predomi-

nating on immunofluorescence microscopy. Reactivities of sera from individuals without disease were determined using 25 blood samples from blood donors.

Results

Enzyme-linked immunosorbent assay (ELISA). The precision and reproducibility of ELISA of antibodies against two of the antigens used in this study, GBM antigens solubilized by collagenase digestion and extracted with guanidine-HCl, respectively, have been reported previously [6, 10]. The coefficient of variation was 6%, mainly due to variable coating and the release of antigens from the polystyrene plates during assay.

Preparations of antigens for ELISA. It was assumed that a large number of antigenic preparations were tested, the chances of discerning specificity for different antigens by the patient's antibodies would be larger. Therefore several procedures were used to solubilize antigens: (1) proteolytic cleavage of nontriple helical proteins by pepsin to solubilize primarily collagen triple helical fragments; (2) specific proteolytic cleavage of triple helical collagen with purified collagenase to liberate proteins other than triple helical collagen; and (3) extraction with guanidine-HCl to release noncovalently bound molecules. Sequential extraction with the different procedures as indicated in Figure 1 was used to allow improved extraction, for instance, after the collagen network of the tissue had been destroyed by collagenase. Furthermore, such procedures may allow selective preparation of purer proteins, better suited for purification of the antigens, which is the ultimate goal of the project. In some extracts proteins were unfolded by reduction and alkylation to alter the number of reactive components and to provide information on the protein nature of antigens. An outline of the protocol for preparation of GBM-antigens to be used for monitoring circulating antibodies is shown in Figure 1. Only solubilized components were used for further studies.

Characterization of antigenic preparations. Using the conditions described, approximately 75% (by weight) of the GBM was solubilized by digestion with bacterial collagenase, approximately 60% by digestion with pepsin, and approximately 50% was extracted with guanidine-HCl. Solubilized macromolecules were characterized after precipitation with ethanol. Small peptides, protease inhibitors, and salts present in digests or extracts are not precipitated and do therefore not interfere in the analyses. The composition, then, reflects larger polypeptides, including those being immunoreactive. The ratio of hydroxyproline to α -amino nitrogen (total protein) indicates the proportions of collagen to total protein in the precipitates, Table 1. Collagenase digests contained mostly noncollagenous proteins and only small amounts of collagen. It is likely that collagenous peptides liberated during digestion are too small to be precipitated with ethanol. Pepsin digests, on the other hand, contained collagen, which was further enriched by salt precipitation used to purify type IV collagen, Table 1. Direct extracts of GBM with guanidine-HCl released noncollagen proteins and some collagen as shown by the intermediary ratios, Table 1. The guanidine-HCl extracts of the residue after collagenase as well as after pepsin digestion contained high proportions of collagen.

Further information on the character of the antigens solubilized by the procedures was obtained by using a rabbit antiserum specific for human type IV collagen [6] as well as a rabbit

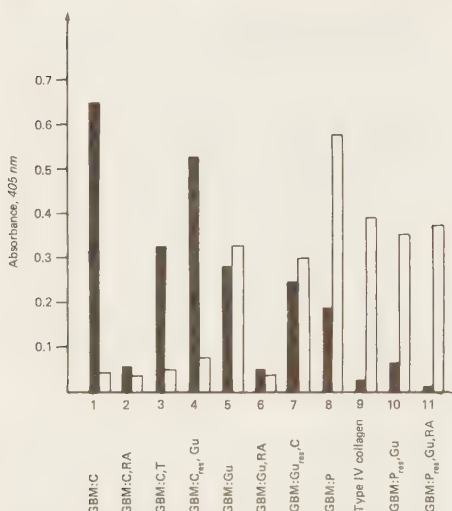


Fig. 2. The reactivity in ELISA of two rabbit antisera with antigens prepared from GBM. The reactivity of a rabbit antiserum (antiglycoprotein), specific for noncollagenous domains of the GBM (black bars) and the reactivity of specific rabbit anti-type IV collagen antiserum (open bars). Absorbance values found with the pre-immune serum from the same animal (background absorbance) were subtracted from the absorbance values found with immune sera.

antiserum specific for noncollagen proteins from GBM (antiglycoprotein) [6]. Previous studies have shown that the antiserum directed against type IV collagen does not react with the small peptides formed upon digestion of type IV collagen with bacterial collagenase [6]. The reactivities with the two antisera of extracts and digests of GBM were determined using ELISA (Fig. 2). Expectedly, all samples digested with collagenase reacted strongly with antiglycoprotein antibodies, while showing no reaction with antitype IV collagen antibodies. Samples digested with pepsin, on the other hand, contained primarily type IV collagen as shown by the strong reaction with the relevant antiserum, while not reacting or reacting only weakly with the antiglycoprotein antiserum. The guanidine-HCl extracts showed variable reactions. Direct extracts of samples not previously digested contained components reacting with antiglycoprotein antibodies as well as with antitype IV collagen antibodies. Collagen triple helical structures of samples treated with collagenase were destroyed and did not react with antitype IV collagen antibodies. The noncollagen proteins on the other hand were destroyed by prior pepsin digestion and did not react with antiglycoprotein antibodies (Fig. 2).

The patterns on SDS-PAGE of the collagenase digest and guanidine-HCl extract of GBM, respectively, were quite complex showing the presence of several components in both preparations (Fig. 3).

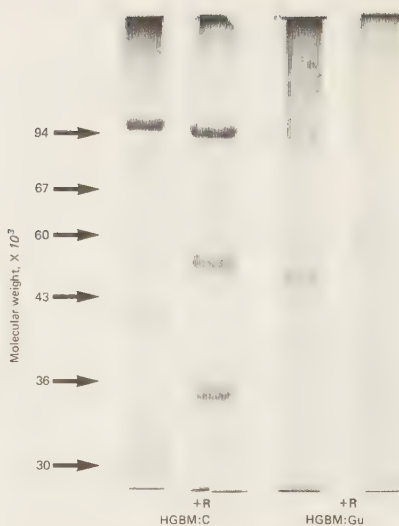


Fig. 3. SDS-polyacrylamide gel electrophoresis (6.25% gel) of antigen preparations precipitated with ethanol. Samples, which were reduced prior to electrophoresis, are indicated by (+R).

ELISA of antibodies in sera from patients. Sera from patients with various disease syndromes reacted differently with the spectrum of antigens (Figs. 4 and 5). Notably, however, sera from the patients with Goodpasture syndrome showed similar reaction profiles. The serum from one patient with Goodpasture syndrome was tested against all the antigen preparations. This serum showed high titers in ELISA with all antigens solubilized by collagenase digestion of GBM, regardless of prior extraction with guanidine-HCl (Fig. 4). It is therefore likely that the antigen or antigens involved in this disease are bound firmly into the matrix of the basement membrane and require extensive cleavage of the collagen network to be released. Antigens solubilized by direct extraction of GBM with guanidine-HCl, however, showed some reactivity with the serum, while pepsin digestion appeared to destroy the antigen(s). None of the samples isolated from pepsin digests reacted with serum from patients with Goodpasture syndrome. The antigens therefore were of a noncollagen nature. Since most of the immune reactivity was lost on reduction and partly by digestion with trypsin (Fig. 4), it appears that the antigen is protein in nature, having a tertiary structure depending on disulphide bridges. Altogether 18 patients with Goodpasture syndrome have had IgG antibodies of variable titers reacting with antigen(s) solubilized by collagenase digestion of GBM (Fig. 5). Ten sera from patients with Goodpasture syndrome showed no reactivity [6], when analyzed for antibodies against preparations solubilized by pepsin digestion (type IV collagen).

Serum from one of the patients having systemic lupus erythe-

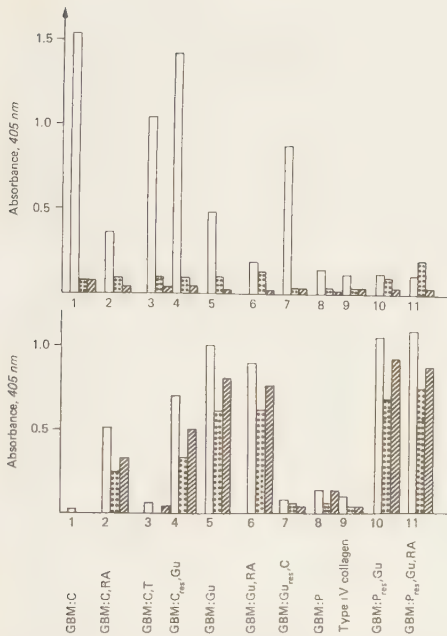


Fig. 4. The reactivities in ELISA of serum from a patient with Goodpasture syndrome (upper panel) and serum from a patient with systemic lupus erythematosus (lower panel) against 11 soluble GBM preparations (indicated in Fig. 1). Symbols are: IgG antibody reactivity (□); IgM antibody reactivity (▨); IgA antibody reactivity (▩). The mean absorbance values found for two normal sera in ELISA were subtracted from the absorbance values found with the patient sera.

matosis with nephritis showed entirely different reactions with the antigens (Fig. 4, lower panel). The antigen(s) could be extracted with guanidine-HCl and was insensitive to reduction. The antigens were not released by digestion with collagenase, but the residue contained reactive antigens which could be solubilized by extraction with guanidine-HCl. Similarly the antigen(s) was present in residues of GBM after pepsin digestion and was extracted with guanidine-HCl (Fig. 4). This antigen(s), then, is not extracted by the enzyme digestions, although the structure of the GBM is altered extensively. It is likely that the antigen(s) is an integral part of the basement membrane rather than being a contaminant. It appears, however, to be bound in the membrane only by noncovalent bonds, which can be dissociated by chaotropic agents, such as guanidine-HCl.

In subsequent experiments sera from patients with several forms of nephritis and having antibasement membrane antibodies have been analyzed for antibody specificity (Fig. 5). All the sera react primarily with antigens solubilized with guanidine-

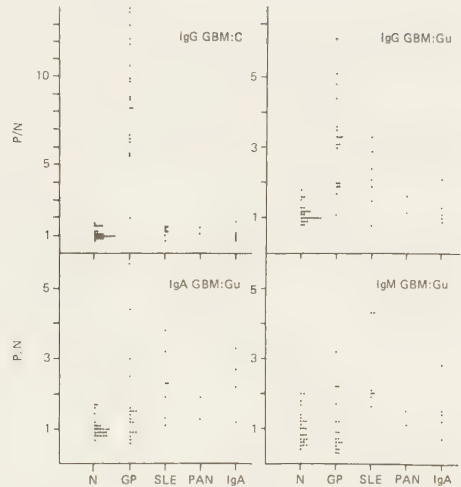


Fig. 5. Circulating antibodies against GBM. Antigens were solubilized with bacterial collagenase (GBM:C) and with 6 M guanidine-HCl extraction (GBM:Gu). Values are expressed as a quotient (P/N) between the absorbance value found for the patient sera and the mean of the absorbance values for three normal sera.

HCl. Subsequent experiments (Fig. 5), using guanidine-HCl extracted antigens, show that patients with systemic lupus erythematosus have variable IgG, IgA, and IgM responses, while patients with IgA-related nephritis primarily have IgA antibodies. A few patients with periarteritis nodosa have variable Ig responses against the guanidine-HCl-extracted antigens (Fig. 5). Normal control subjects show insignificant reactivity against GBM antigens tested. It should be stressed, that so far, no patient has had circulating antibodies directed against antigens in the pepsin digest, that is, against type IV collagen.

Discussion

Isolated basement membrane contains several components potentially causing an antibody response. Chemically distinguishable structures are the triple helical collagen domains and the nontriple helical protein domains, the latter probably being glycoproteins and proteoglycans [17–19]. The two types of structures contain a number of different components all bound together via covalent bonds or noncovalent molecular interactions [16, 17]. Little is known of distinct components although a few, that is, type IV collagen [17], laminin [20], 7s collagen [21], and proteoglycans [19], have been identified. Animals immunized with preparations of GBM develop a spectrum of antibodies directed against several components [11, 22]. It has been shown that antipeptide IV collagen antibodies as well as several types of antiglycoprotein antibodies are produced in response to such immunization [11, 22]. Some animals develop glomerulonephritis mediated by the antibodies [11]. There are several

problems in understanding such a model disease. The antigen preparation may contain several components that have become antigenic due to changes in structure as a result of the preparation procedure. Spontaneous anti-GBM antibody associated nephritis on the other hand seems to occur only in humans [1]. Reports on the character of the antigens are conflicting and collagen peptides, oligosaccharide side chain, as well as noncollagen proteins, have been implicated [4, 7, 23]. Most preparations studied, however, have been crude and the nature of the true antigens is dubious.

The strategy chosen in the present investigation has been to use several preparations of components solubilized from GBM to select that preparation containing the antigen relevant to a particular form of nephritis. The aim is to use such a preparation for purification of components reacting with the antibodies present in the patient serum. The different extracts overlap with regard to antigen contents. It should be stressed that it is probably essential to include N-ethylmaleimide in extraction solvents, particularly when guanidine-HCl is used, to avoid disulphide exchange causing problems in the subsequent purification of antigens.

An important observation in the present study is the different character of the antigen(s) involved in Goodpasture syndrome as compared to those involved in other forms of nephritis. Although both types of antigens represent structures not containing the triple helical portions of collagen, they are markedly different with respect to sensitivity to chemical modifications. Only the antigen reacting with the IgG antibodies present in sera from several patients with Goodpasture syndrome is sensitive to reduction.

The localization of the different antigens in the GBM remains essentially unknown. The present study may, however, provide some clues. Good yields of antigens reacting with antibodies in sera from patients with Goodpasture syndrome was obtained only with drastic destruction of the structure of the basement membrane using collagenase digestion. This finding may be expected since immunoelectron microscopy [24] and epi-immunofluorescence microscopy [25] indicate that the antigenic sites are located in the lamina densa. The pathogenic significance of the antibodies present in sera from patients with systemic lupus erythematosus or periarthritis nodosa is more doubtful. Nephritis has usually been ascribed to circulating immune complexes, frequently observed [1]. Partially linear patterns of immunoglobulins and complement have, however, been observed, indicating a more complex genesis [26]. Furthermore, in more recent studies circulating antibodies of IgG directed against GBM-antigens have been demonstrated using RIA [8].

The demonstration of circulating antibodies directed against glomerular basement membrane antigens, indicates that such antibodies may be involved at least in the progression of the disease. The antigens are extracted with guanidine-HCl and are bound via noncovalent bonds into the basement membrane. According to Huang [27], only basement membrane structures of endothelial and mesangial origin change upon treatment with guanidine-HCl. It is therefore possible that the antigens reacting with IgA, IgM and/or IgG antibodies in sera from patients having other forms of nephritis are located in lamina rara or in mesangial regions and are of endothelial origin.

In systemic lupus erythematosus antibodies against various auto-antigens, including nuclear antigens, are common. The

antibodies studied in this report, however, appear to be directed against basement membrane structural components. As discussed above, it appears that the antigens detected are anchored tightly in the basement membrane and therefore probably do not represent extraneous molecules. Further support for the contention that these antigens represent basement membrane components, rather than cellular material, was provided by the identical reactivities of extracts of GBM preparations made by the procedure of Meezan et al [28] which includes extraction with detergents and digestion with DNA'se (data not shown). Furthermore, the serum from one patient with systemic lupus erythematosus had high anti-DNA antibody titers, but did not react in the ELISA against GBM-antigens.

Another important observation is the presence in sera from patients with IgA nephritis [29] of IgA antibodies reacting most markedly with antigens present in guanidine-HCl extracts of GBM. The etiology of IgA nephritis is unknown and subgroups where auto-immune antibodies have pathophysiological significance may exist.

By using antigens solubilized with collagenase, an anti-GBM antibody assay specific for Goodpasture syndrome is obtained. In contrast, antigens solubilized with chaotropic agents, although reacting with sera from patients with Goodpasture syndrome, will also react with sera from patients with other forms of renal disease. Therefore, the clinical specificity of anti-GBM antibody assays is highly dependent on the antigen preparations used. Future studies using purified antigens may provide further diagnostic aid and show that the specificity of the antibodies may vary with the disease.

Acknowledgments

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GOODPASTURE'S SYNDROME

Isolation of the specific glomerular basement membrane antigen

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Footnote:

Abbreviations used: GBM - glomerular basement membrane

GP - Goodpasture's syndrome

GP-antibodies - antibodies present in patients
with GP and directed against
GBM

GP-antigen - antigen reacting with antibodies
from patients with GP-syndrome

SDS-PAGE - sodium dodecyl sulphate polyacryl-
amide gel electrophoresis

ABSTRACT

In anti-GBM antibody-associated glomerulonephritis with or without alveolar necrosis, commonly referred to as Goodpasture's syndrome, there is a formation of autoantibodies against the basement membrane of kidney and lung. The antigen involved was purified from human glomerular basement membrane digested with highly purified clostridial collagenase. Initial fractionation of the digest on Sephacryl S-200 was followed by affinity chromatography using anti-human glomerular basement membrane antibodies covalently bound to Sepharose CL-4B. Such antibodies do not react with the relevant antigen, while they react with several other components in the basement membrane. The antigen(s) were finally purified by chromatography on Sephacryl S-200 eluted with 6M guanidine-HCl. The purified nonreduced sample contained two components with closely similar mobilities on SDS-polyacrylamide gel electrophoresis. After reduction they moved as one component, corresponding to a molecular weight of 26 000. The purity was further verified by chromatography on CM-cellulose, where the antigen eluted as one, slightly asymmetric peak.

Immunologically identical aggregates of higher molecular weight (i.e. 48 000) were also present in the crude digest. Reduction of purified such aggregates released some protein with a molecular weight of 26 000 but a large proportion was insensitive to reduction. Seven patients with Goodpasture's syndrome, all had circulating antibodies directed only against the purified antigen.

INTRODUCTION

Anti-GBM antibodies are most commonly associated with the triad of glomerulonephritis, alveolar necrosis and linear deposits of immunoglobulins along the basement membranes of glomerulus and lung (1). The antibodies involved bind to the GBM, with ensuing complement activation. Attracted leucocytes may release proteases that have the capacity to degrade the GBM. The participation of anti-GBM antibodies in the events resulting in destruction of basement membrane in GP is well established, although reports on the nature of the basement membrane antigen have been conflicting.

The typical clinical syndrome was first described by Goodpasture (2) and is therefore often referred to as Goodpasture's syndrome. More recently variant cases not having the typical clinical picture have been described (1). Nevertheless also these cases are referred to as Goodpasture's syndrome (1).

A major constituent of GBM is the type IV collagen molecules (3,4). During the last few years, a number of other distinct basement membrane macromolecules i.e. laminin (5), heparan sulphate proteoglycans (6), entactin (7) and 7S collagen (8) have been isolated. It has been suggested (4) that the basement membrane consists of a regular network of type IV collagen molecules. The 7S collagen, which is released on collagenase digestion, is made up of one of the terminal regions of each of four type IV collagen molecules and forms a domain that contains disulphide bond cross-links between individual collagen chains. The collagen molecules are also bound, one to another, by their other terminal, non-triple helical, collagenase resistant region.

Several attempts have been made to characterize the basement membrane antigen involved in GP. Already in 1951 Krakower and Greenspoon (9) indicated a nephrotoxic antigen localized in the glomeruli. In 1971 McIntosh and Griswold (10) suggested that the antigen is collagen in nature, based on their finding that kidney sections treated with collagenase did not bind antibodies present

in GP-serum. On the other hand, Marguardt et al (11) could show, using in vitro techniques, that the antigen was a non-collagenous glycoprotein since collagenase digestion did not abolish binding of antibodies from patients. In an assay designed by Mahieu et al (12), binding of antibodies from patients with GP to a mixture of antigens extracted from basement membranes could be inhibited using a preparation of disaccharides from collagen. The authors concluded that the antigen was collagenous in origin.

Other sets of findings are the observation that GP sera react neither with type IV collagen nor with laminin (13), and the contradictory observation that the antibodies are indeed directed to laminin and type IV procollagen (14). In previous studies (15) we have shown that antibodies from patients with GP react with antigen solubilized from GBM with collagenase but not with antigen solubilized with pepsin i.e. type IV collagen. Our data, then, indicate that the antigen is non-collagenous in nature. Furthermore Hunt et al (16) have isolated three antigenic fractions of non-collagenous origin using affinity chromatography of collagenase digests of basement membrane on a column containing bound GP-antibodies.

One problem in studying glomerulonephritis with associated anti-basement membrane antibodies is the frequent presence of circulating antibodies directed against non-collagenous basement membrane components in several other forms of glomerulonephritis (17). Previous studies, however, indicate that the antibodies present in GP-patients, react with a unique antigen (17) present in all basement membranes studied (including GBM and lung basement membrane), while no sera from other diagnostic subclasses react with this antigen (unpublished). An assay using preparations of this antigen would therefore allow specific diagnosis of GP.

Furthermore the identification and characterization of the antigen may provide important background information for the understanding of the pathogenesis of glomerulonephritis and

alveolar necrosis.

MATERIALS AND METHODS

Patients; diagnostic criteria

All seven patients had rapidly progressive crescentic glomerulonephritis. One young male patient had serious lung purpura with massive hemoptysis while lung involvement was less pronounced in the other. Immunofluorescence staining of biopsy specimens showed the typical linear pattern of IgG deposition along the GBM in all patients and along lung basement membrane in the one patient with severe lung involvement. The presence of circulating anti-GBM antibodies was verified by ELISA. All the patients survived but none of them recovered renal function in spite of intense treatment with plasma exchange and/or immunosuppressive drugs.

Preparation of glomerular basement membrane

Human kidneys were obtained at autopsy performed within 16 h after death and stored frozen until processed. The adaptation by Westberg and Michael (18) of the method of Spiro (19) and further modified as described (20) was used for the preparation of glomeruli and basement membrane. All preparative steps were done using ice cold, aqueous wash solutions containing the protease inhibitors 4 mM N-ethyl-maleimide, 1 mM phenylmetan-sulfonyl fluoride, 5 mM benzamidine-HCl, 25 mM 6-aminohexanoic acid and 10 mM EDTA. Isolated glomeruli were sonicated to remove cellular material. The remaining insoluble basement membrane was lyophilized and stored dry.

Digestion with collagenase

The collagenase used (Worthington CLS) was purified according to Lee-Own and Anderson (21). The purified collagenase was assayed for remaining non-specific protease using ^3H -acetylated hemoglobin (22). In the presence of the protease inhibitors

listed above, omitting EDTA, no proteolytic activity could be demonstrated in the purified enzyme.

Using a Potter-Elvehjem homogenizer, GBM was suspended at 10 mg/ml in 0.05 M HEPES, pH 7.5, 0.01 M CaCl_2 , 0.05% sodium azide containing the protease inhibitors listed above, omitting EDTA. Collagenase was added at a ratio to GBM of 1:50 w/w and digestion was performed at 37°C for 72 h (23). The solution was centrifuged at 105 000 xg for 1 h to remove all particulate matter and the supernatant was stored in the cold (+4°C) awaiting further analysis. To determine the kinetics of the digestion, samples were withdrawn at different time intervals.

ELISA

The assay for the GP-antigen was essentially the ELISA already described (15,17) with minor modifications. During the progress of this work it was found that coating is more efficient in 6 M guanidine-HCl than in other coating buffers, possibly by the prevention of aggregation of antigen. Furthermore, inclusion of guanidine-HCl to make all solutions 0.6 M was found to increase the degree of inhibition and thereby the sensitivity of the assay.

Chromatography

The proteins solubilized from the basement membrane by collagenase digestion were fractionated on a column (2.4 x 150 cm) of Sephacryl S-200 (Pharmacia Fine Chemicals, Uppsala, Sweden), eluted with 0.05 M Tris-HCl pH 7.5, also containing 0.15 M NaCl, 0.001 M EDTA and 0.05% NaN_3 , 6 ml fractions were collected. After analyses, those fractions containing GP-antigen were pooled. The antigen was further purified by affinity chromatography on a column of Sepharose CL-4B in 0.05 M Tris-HCl pH 8.0, 0.5 M NaCl, to which rabbit IgG directed against collagenase and rabbit IgG directed against particulate human GBM had been coupled using cyanogen bromide (24). The material not bound to the affinity column was

concentrated by ultrafiltration using a UM 2 filter (Amicon Corp, Lexington, Mass. USA), dialyzed against 6 M guanidine-HCl, 0.05 M Tris-HCl pH 7.5 and chromatographed on a column (2.7 x 150 cm) of Sephacryl S-200 eluted with 6 M guanidine-HCl, 0.05 M Tris-HCl pH 7.5. The fractions (each 10 ml) containing GP-antigen activity were pooled and dialyzed against 8 M Urea, 0.04 M NaAc, pH 4.8 and chromatographed on a column of CM-cellulose (Whatman Ltd, Maidstone, Kent, England) (1 x 10 cm) equilibrated in the same buffer. A linear gradient of 0-0.3 M NaCl was used for elution and 8 ml fractions were collected.

Electrophoresis

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was done as described by Laemmli (25), in 10%, 12%, 14% or 16% gels. Gels were stained with Coomassie brilliant blue R-250 (Merck, Darmstadt, West-Germany). Proteins in aliquots of the fractions to be analyzed were precipitated with 10 volumes of ethanol containing 10 mM sodium acetate before electrophoresis. In some instances gels were stained with silver as described by Morrissey (26). Western blotting was performed as described by Burnette (27). Proteins separated on 12.5% SDS-PAGE were blotted onto nitrocellulose paper (Bio Rad Laboratories, Richmond, USA). Bound proteins were detected by incubation of the papers with diluted (1/50) serum from a GP-patient for 1 h, followed by incubation with anti-human IgG labelled with horseradish peroxidase (Dakopatts, Copenhagen, Denmark) for 1 h. Peroxidase activity was detected using 3-amino-9-ethyl-carbazole as the substrate.

Modification of antigens

The purified GP-antigen was fragmented by digestion with DCC-treated Trypsin (Sigma Chemicals, Missouri, USA) at a ratio of enzyme to substrate of 1:50 in 0.1 M Tris-HCl, pH 8.0 for 4 h at 37°C. In other experiments the GP-antigen was unfolded by

reduction with dithiothreitol and alkylated with iodoacetic acids as described elsewhere (17).

RESULTS AND DISCUSSION

Collagenase digestion

The purified collagenase was used to specifically degrade the triple helical parts of the GBM thereby releasing the intact GP-antigen. The digestion of the GBM was continued for 72 h (23) in order to obtain a limit digestion of the collagenous portions of the membrane. Under these conditions 70-80% of the membrane (w/w) was solubilized. Kinetic studies showed that solubilized GP-antigen increased rapidly during the first 4 hours and then increased more slowly until 72 hours. It appeared that extended digestion did not destroy the released antigen, since even on prolonged digestion the GP-antigen activity did not decrease.

Isolation of the Goodpasture's antigen

The proteins solubilized by the collagenase digestion were fractionated by gel chromatography on Sephacryl S-200, fig. 1. The chromatogram showed several peaks, one in the void volume (S 200 I), an early retarded peak having a K_{av} of about 0.17 (S 200 II) followed by a minor peak with a K_{av} of 0.33 (S 200 III). More UV-absorbing material eluted late but contained no GP-antigen. The major portion of the GP-antigen eluted in the S 200 III peak, but appeared not to be homogenous, fig. 1 a. Additional GP-antigen was eluted in the first and second peak, (S 200 I and II), possibly a result of aggregation of components or incomplete degradation of the membrane. The first peak (S 200 I) contained various undefined basement membrane components as shown by strong reaction with an antiserum raised in rabbits against intact glomerular basement membrane (not shown). The second peak contained among other components, albumin derived from serum contaminants and the collagenase used for digestion, fig. 1 b. To obtain a better idea of the number of components present,

samples of selected fractions were separated by 12.5% SDS-PAGE, (not shown). Fraction S 200 III, contained major components with mobilities corresponding to molecular weights of about 48 000 and 26 000, possibly representing the GP-antigen. These components were also present in fraction S 200 II.

To further purify the GP-antigen the pooled fraction S 200 III was passed through a affinity column containing bound anti-collagenase antibodies and anti-GBM antibodies. The latter were raised in rabbits against particulate human GBM and did not react with the GP-antigen. The anti-GBM antibody preparation in addition contains antibodies to plasma proteins, which constitute 1-2% of the isolated membrane (11). Such contaminating plasma proteins were, however, removed by the affinity column. The material not bound to the immunosorbent column contained 95% of the chromatographed GP-antigen while neither albumin nor collagenase could be detected using ELISA. Electrophoresis of this material on SDS-PAGE (not shown) showed four components with apparent molecular weights of about 60 000, 48 000, 26 000 and 20 000. Since these components were well separated in the denaturing conditions of the SDS-PAGE, an attempt was made to further purify the antigen by gel chromatography under the dissociative conditions of 6 M guanidine-HCl. The material not bound to the affinity column was concentrated by ultrafiltration (UM 2) and subsequently dialysed against 6 M guanidine-HCl, 0.05 M Tris-HCl, pH 7.4. The proteins were then chromatographed on a column of Sephacryl S-200 eluted with the same buffer (Fig. 2). Two protein peaks plus UV-absorbing material eluting in the total volume could be seen. More than 96% of the GP-antigen chromatographed in the second peak (II) while the remaining activity chromatographed in the first peak (I). The proteins in the peaks were further identified by SDS-polyacrylamide gel electrophoresis. Peak II contained only one component having an apparent molecular weight of 26 000 and the material in this peak was considered as pure GP-antigen. The overall yield of the

GP-antigen represents about 10% of the proteins in the collagenase digest used for purification. The component present in peak I had a mobility corresponding to a molecular weight of 48 000. It was not further analyzed.

Characterization of the GP-antigen

The purity of the GP-antigen was tested by chromatography on a column of CM-cellulose equilibrated in 8 M Urea, 0.04 NaAc, pH 4.8. Proteins were eluted with a salt gradient from 0-0.3 M NaCl (Fig. 3 a). A small amount of UV-absorbing material did not bind to the column. This material contained no reactive antigen nor was any protein detected on SDS-PAGE. The major portion of the protein eluted with about 0.2 M NaCl. This peak showed some microheterogeneity and the fractions eluted at higher ionic strength had a higher relative content of GP-antigen.

SDS-PAGE of nonreduced samples showed the presence of two components, Fig. 3 b. The particular preparation shown, also contained a minor component (less than 1%) with a somewhat higher mobility on SDS-PAGE (fractions 18 and 20, Fig. 3 b). Western blotting using serum from a GP-patient showed only one component, Fig. 3 c, with a mobility corresponding to the slower moving band. After reduction the two components had the same mobility, Fig. 3 d. It is possible that the two components observed represent closely similar structures, perhaps originating from the globular portions of two types of type IV collagen chains, as is discussed below. Only one of the components appear to represent the antigen.

Relationship of activity to structure

In an attempt to learn more of the structure of the GP-antigen, it was reduced and alkylated in 6 M guanidine-HCl to demonstrate presence of a tertiary structure depending on disulphide bonds. The reactivity in ELISA was completely inhibited (Fig. 4) showing that the antigen contains intrachain disulphide bonds which are essential for the GP-antigen activity. Supporting evidence

is the absence of reactive antigen in Western blots of samples reduced prior to SDS-PAGE (data not shown).

Digestion with trypsin (Fig. 4) reduced the reactivity in the ELISA showing the protein nature of the antigen and also that the whole structure is not required for antigenicity. SDS-PAGE of these samples showed that all the GP-antigen had been cleaved.

Structure and composition

To determine the molecular weight of the GP-antigen, SDS-electrophoresis of reduced samples and reference proteins was performed on gels having different concentrations of acrylamide (10%, 12%, 14% and 16%). A Ferguson plot (28) was constructed by plotting the relative migration distance for all proteins electrophoresed against the different concentrations of acrylamide (not shown). The line for the GP-antigen extrapolated to the same point as the reference proteins showing that the GP-antigen behaved ideally in the system. Its molecular weight was calculated to be 26 000.

The amino acid composition of the GP-antigen is shown in table I. Interestingly the antigen contained only traces of hydroxyproline and no hydroxylysine. Furthermore, its content of glycine is only half that of collagenous proteins. It is therefore highly unlikely that the GP-antigen is part of the triple helical (collagenous) portions of the type IV basement membrane collagen.

Higher molecular weight structures containing the GP-antigen

As is discussed above other components, that react with serum from patients with Goodpasture's syndrome elute less retarded on gel chromatography than the GP-antigen, i.e. fractions S 200 I and S 200 II, Fig. 1. Such heterogeneity of the antigens in collagenase digests of GBM has previously been shown by Holdsworth et al (29) and Hunt et al (16). Antigens having apparent molecular weight of 27 000 and 53 000 (29) or 25 000 to

200 000 (16) were demonstrated.

The nature of the antigens present was further studied. Components in fractions of S 200 I were not dissociated to smaller structures by SDS as shown by SDS-PAGE followed by Western blotting (data not shown). It is possible that this fraction mainly contains GP-antigens only partially solubilized by the digestion with collagenase. Components in fraction S 200 II, on the other hand, could be dissociated both with SDS and with 6 M guanidine-HCl to yield smaller molecules with a high activity in the GP-antigen assay. Chromatography of this fraction on Sephacryl S-200 eluted with 6 M guanidine-HCl, Fig. 5, gave two peaks containing antigen eluting at the same positions as the two peaks obtained when fraction S 200 III was chromatographed under identical conditions, compare Fig. 2. SDS-PAGE further verified that also the fractions recovered from S 200 II had apparent molecular weight of 48 000 and 26 000, respectively, Fig. 5. A portion of the material in peak I (indicated in Fig. 5) could be dissociated by reduction to yield the 26 000 dalton component (data not shown). By using ELISA the similar character of the different antigens was further demonstrated. Antibodies present in serum from a patient with GP react with the antigens in pools I and II (cf. Fig. 5), and with the GP-antigen (pool II, Fig. 2). The binding was in all cases completely inhibited by the purified GP-antigen, i.e. component II from fraction S 200 III, Fig. 6. Conversely it was also shown that binding of antibodies to the GP-antigen was inhibited by the other components (data not shown). It appears then, that the components with apparent molecular weights of 48 000 as well as the components with apparent molecular weight of 26 000 contain identical antigenic determinants.

It is possible that the larger molecules represent complexes of the GP-antigen. Support for this hypothesis was provided by amino acid analysis of the 48 000 dalton component isolated from S 200 II (Fig. 5), table I. Its composition is indeed similar to that of

the purified GP-antigen.

The molecular origin of the GP-antigen is not disclosed by the present investigation. It's amino acid composition, however, is rather similar to that of the collagenase resistant non-triple helical, NC1 portion of the type IV collagen. Furthermore the size of the smallest component containing the GP-antigen is rather similar to that of the NC1 (4). It is possible that the various aggregation forms of the GP-antigen demonstrated, may represent monomer, dimer and trimer of the NC1 peptide portion of type IV collagen.

Antibody specificity of different patients

Proteins in collagenase digests were separated by SDS-PAGE and blotted onto nitrocellulose paper. Sera from 7 patients with Goodpasture's syndrome were incubated with strips of the paper. Subsequent labelling with anti-human IgG-peroxidase conjugate allowed demonstration of bound immunoglobulins from patients, Fig. 7. All patients gave the same patterns, showing strongest reactions with components with electrophoretic mobilities corresponding to 26 000 dalton (i.e. the GP-antigen) and 48 000 dalton.

The staining depends on the presence of IgG, since serum passed through a column of protein A-Sepharose did not stain the blot. Furthermore, the antibodies could be eluted from the protein A-Sepharose and used for staining, Fig. 7.

In conclusion, patients with Goodpasture's syndrome have circulating antibodies directed to one type of antigen, probably present in the basement membrane in monomeric as well as in aggregated forms. This antigen can also be demonstrated in basement membrane preparations from lung and placenta (unpublished observations). The antigen then provides an immunological marker of this disease, because patients with other forms of glomerulonephritis have no circulating antibodies to the GP-antigen while they may have antibodies to other GBM structures (17).

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<u>Goodpasture antigen</u>		
<u>Amino acids*</u>		
<u>(residues/1000 residues)</u>		
	26 K	48 K **
3-hydro	n.d.	n.d.
4-hydro	2.3	8.6
ASP	88.0	84.5
Thr	58.2	55.6
Ser	104.2	106.1
Glu	144.2	151.8
Pro	82.9	72.6
Gly	130.5	143.7
Ala	80.5	74.9
Cys	3.0	9.8
Val	35.7	36.7
Met	n.d.	n.d.
Ile	31.3	28.8
Leu	69.1	63.7
Tyr	25.2	27.3
Phe	39.9	32.7
His	27.5	30.4
Hylys	n.d.	n.d.
Lys	28.6	26.4
Arg	48.9	46.4

n.d. = not detected

*) Analysis was performed after hydrolysis of samples
in 6 M HCl for 24 h at 110°C.

**) Component I from S 200 II (Fig. 5)

Figure 1. Chromatography on Sephacryl S-200 of glomerular basement membrane components solubilized by digestion with collagenase. Elution with 0.15 M NaCl, 0.05 M Tris-HCl, 0.001 M EDTA, 0.05% NaN₃, pH 7.5. Proteins were quantified by ELISA using serum from a patient. Collagenase and albumin were quantified using ELISA

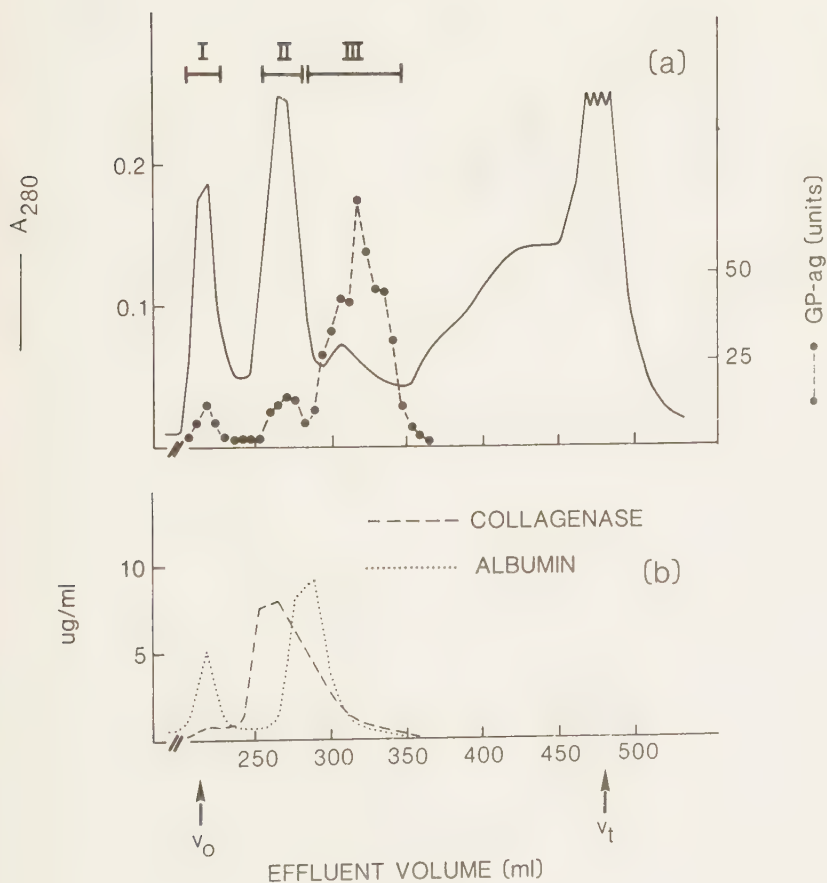


Figure 2. Chromatography on Sephacryl S-200 of affinity purified GP-antigen under dissociating conditions. The proteins were chromatographed in 6 M guanidine-HCl, 0.05 Tris-HCl pH 7.5. The fractions were analysed for their content of protein by absorbance at 280 nm and for content of GP-antigen by ELISA. Proteins in aliquots of fractions, indicated by I and II, were electrophoresed on 15% SDS-polyacrylamide gels under non-reducing conditions. Molecular weight markers, however, were reduced, prior to electrophoresis.

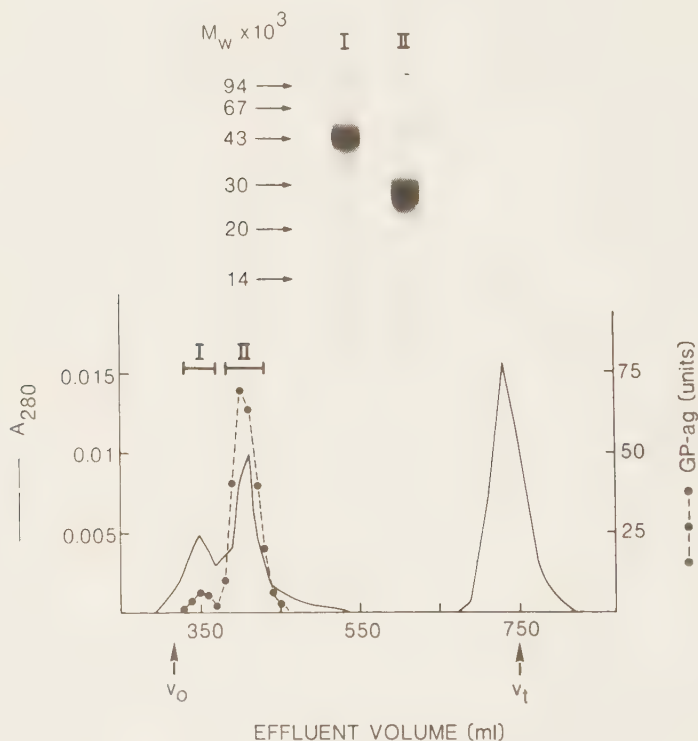


Figure 3. Ion exchange chromatography in 8 M urea on CM-cellulose of the GP-antigen (pool II, Fig. 2). A gradient of 0 to 0.3 M sodium chloride in 8 M Urea was used for elution. The fractions were analyzed (a) for content of protein by absorbance at 280 nm and for content of GP-antigen using ELISA. Proteins in samples of every second fraction, eluted at volumes from 112 to 160 ml, were precipitated with ethanol and electrophoresed on 12.5% polyacrylamide gels with (b) and without (d) prior reduction. Proteins were stained with silver (26). Duplicate, non-reduced, samples of the same electrophoretogram were blotted on to nitrocellulose paper and stained using GP-antigens (c).

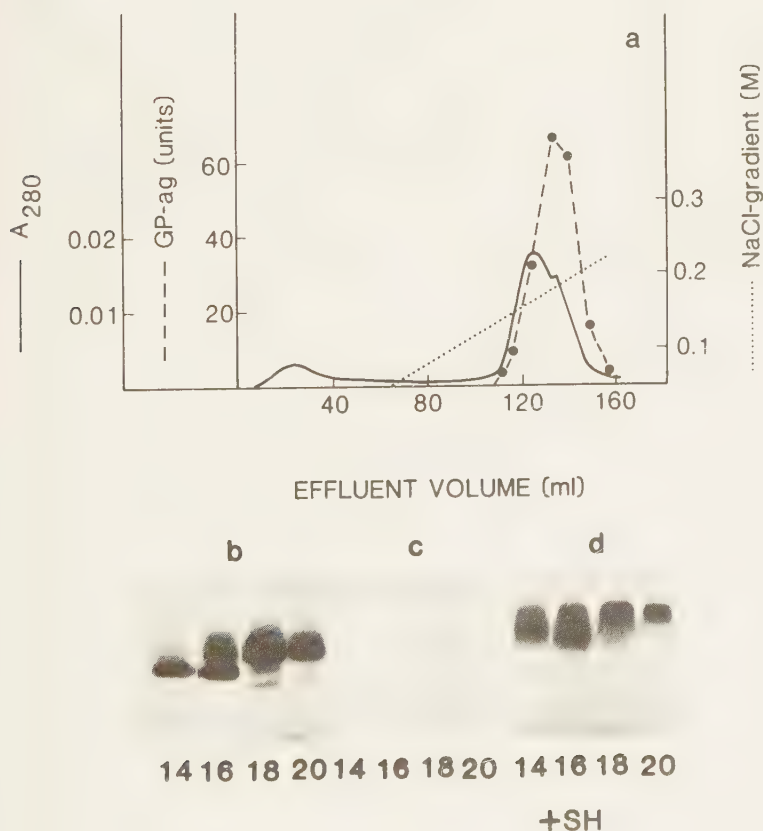


Figure 4. The dependence of intact disulphide bonds for antigenic activity. The purified GP-antigen was modified by reduction and alkylation (■—■) or by degradation with trypsin (○—○) and allowed to inhibit the ELISA of the GP-antigen. The curve for the intact GP-antigen (●—●) is shown for comparison.

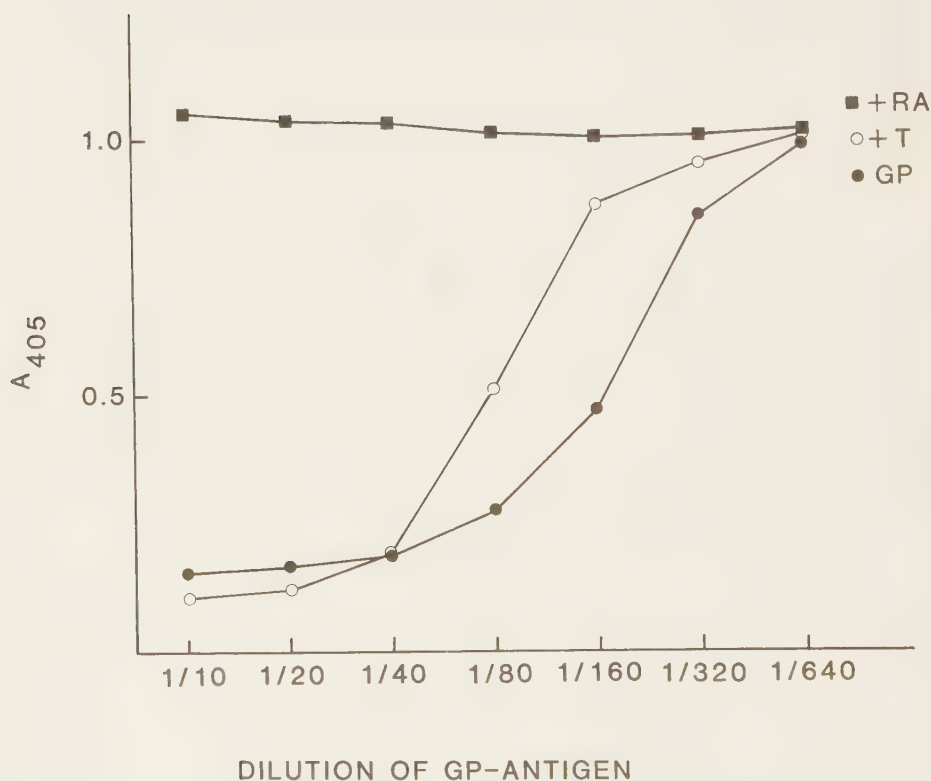


Figure 5. Chromatography on Sephacryl S-200 of the larger molecular size Goodpasture antigen recovered in peak II (Fig. 1). The material was dissolved in 6 M guanidine-HCl which was also used for elution. Fractions were pooled as indicated and electrophoresed on 15% SDS-polyacrylamide gels under non-reducing conditions. Molecular weight markers, however, were reduced, prior to electrophoresis.

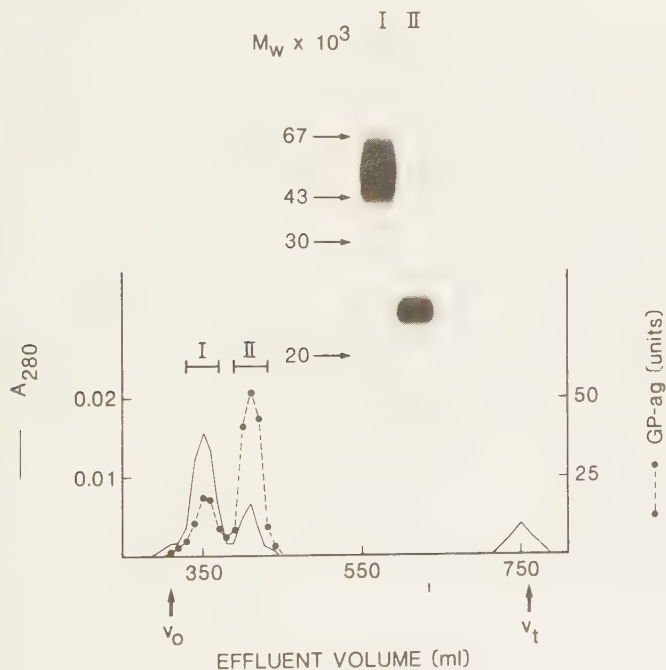


Figure 6. Crossreactivity of GP-antibodies with different antigenic preparations shown by a competition ELISA. Fractions I (o---o) and II (Δ ··· Δ) in Fig. 5, and fraction II ($\bullet$ --- $\bullet$) in Fig. 2 were coated on polystyrene microtiter wells. Binding of GP-antibodies to coated antigen was inhibited by addition of the purified GP-antigen (fraction II in Fig. 2) at indicated dilutions.

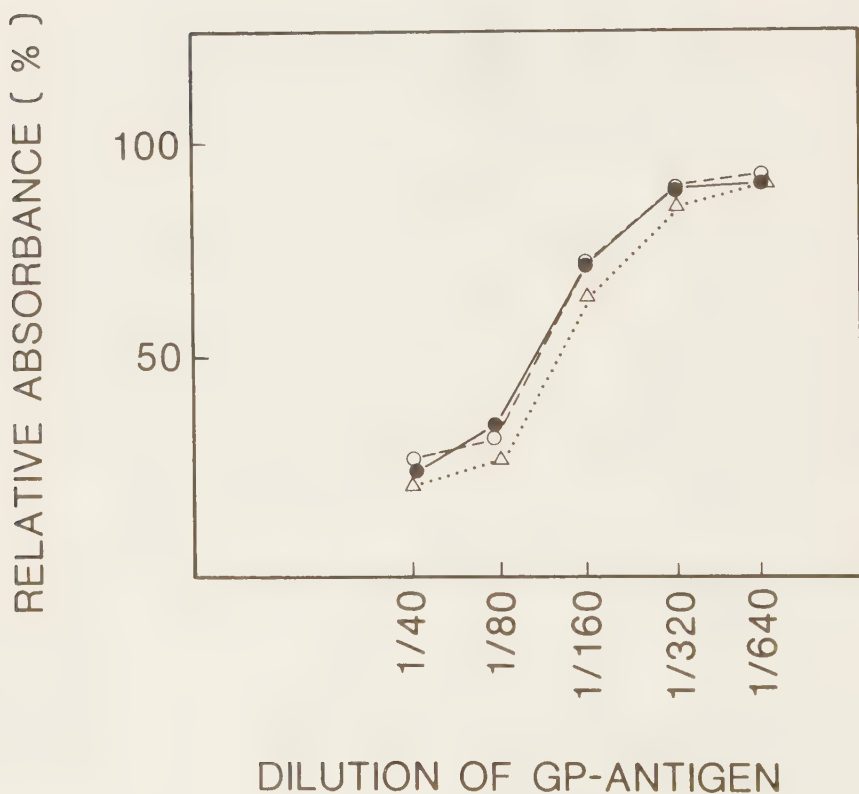
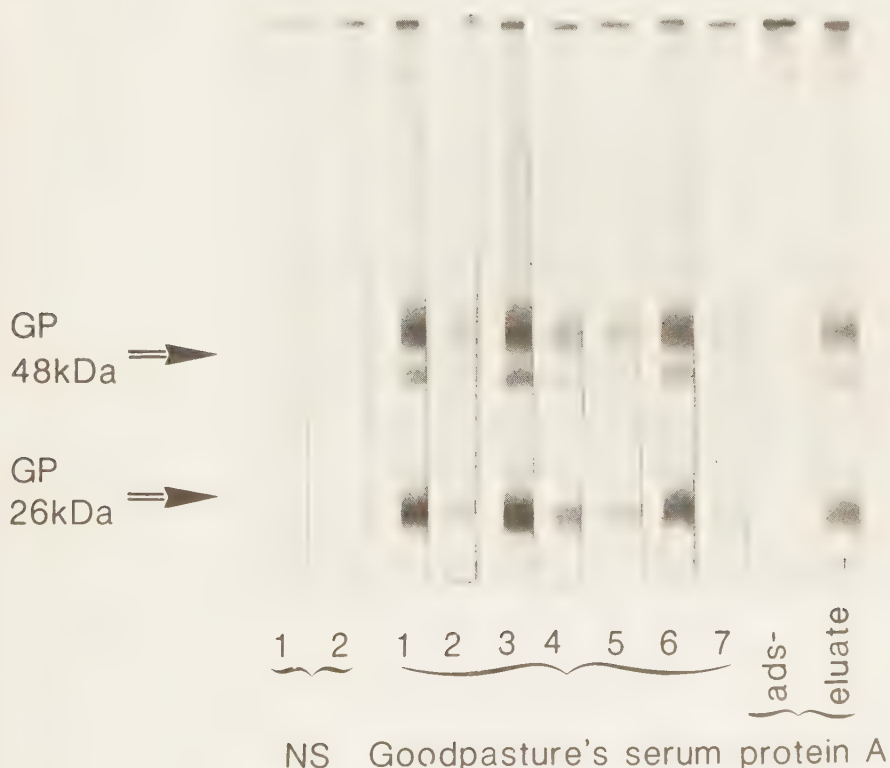


Figure 7. IgG of GP patients showing binding to the same proteins. Digests of GBM with collagenase were fractionated by SDS-PAGE. Proteins were blotted on to nitrocellulose paper and stained using serum from 7 patients with Goodpasture's syndrome, 2 control sera (NS) and one Goodpasture serum after adsorption of IgG using protein A-Sepharose (prot A ads). An eluate of the antibodies bound to the protein A-Sepharose, was also used for staining (prot A eluate).



Goodpasture's Antigen of the Glomerular Basement Membrane:
Localization to Noncollagenous Regions of Type IV Collagen

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Abstract. The glomerular basement membrane antigen in Goodpasture's syndrome is a collagenase-resistant molecule with a molecular weight of about 26,000. Type IV collagen isolated from glomerular basement membrane contains collagenase-resistant sequences in its structure. Polyacrylamide gel electrophoresis, enzyme linked immunosorbent assay, and chemical analysis were used to demonstrate that the collagenase-resistant sequences of type IV collagen contain Goodpasture's antigen.

Approximately 5% of the cases of glomerulonephritis are mediated by autoantibodies to glomerular basement membrane (GBM) (1-2). Most of these patients present with Goodpasture's syndrome which is characterized by glomerulonephritis and alveolar necrosis. The disease has attracted great interest as a model for autoimmune disease in man. Classically, Goodpasture's antibodies have been

demonstrated by immunofluorescence and appear to be distributed in a uniform manner along the GBM (3). More recently radioimmunoassay (4-5) and enzyme linked immunosorbent assay (ELISA) (6) have been used for the detection of circulating antibodies directed against GBM antigens. Evidence suggesting a strong immunogenetic susceptibility to this disease (7) may reflect the activity of an immune response gene. If so, it is likely that the immune response is restricted to one or a few epitopes of a complex antigen. Identification of the Goodpasture's antigen is thus an essential step in further understanding the exact nature of this disease and in the design of specific therapy. We describe research which demonstrates that the Goodpasture's antigen is localized in the type IV collagen constituent of the GBM.

The Goodpasture's antigen has been identified in collagenase digests of human GBM (6,8-10). Subsequent purification showed that the antigenic activity is contained in two noncollagenous polypeptides, the smaller having a molecular weight of 26,000 (9-11). A higher molecular weight form (50,000) is also present (9-10) and can be partly cleaved to yield the 26,000 dalton component by reduction of disulfide bonds. However, reduction results in loss of antigenicity (11). The molecular properties of the Goodpasture's antigen suggest it may be located in type IV collagen of the GBM.

Type IV collagen is now known to be composed of two proteins having molecular weights of about 180,000 (α_1 type IV collagen) and 170,000 (α_2 type IV) (12-15). In the intact GBM these proteins form a discontinuous triple helix which is stabilized by disulfide bonds (16-17). Collagenase digestion of purified α_1 type IV collagen from bovine GBM results in resistant product(s) of molecular weight 28,000, as revealed by SDS-PAGE in a continuous buffer system. SDS-PAGE in a discontinuous buffer system resolves this component into two proteins with observed molecular weights of 31,000 and 33,000 (12). Therefore,

it is plausible that the 26,000 dalton collagenase-resistant product from human GBM which binds the Goodpasture's antibody is identical to the collagenase-resistant products derived from the alpha₁ type IV molecule.

In order to test this hypothesis and to use bovine GBM, the species from which numerous structural studies have been performed, it was necessary to establish that it contained the Goodpasture's antigen. Bovine GBM (18) was digested with collagenase and the products were examined by SDS-PAGE (Fig. 1, gel 1-3). Two products were observed with apparent molecular weights of 22,000 and 45,000. These molecular weights are similar to those of collagenase-resistant products which react with Goodpasture's antibodies from human GBM (9-11). The two products were isolated by chromatography on Bio-gel P-100 and were then analyzed prior to and after reduction of disulfide bonds by SDS-PAGE (Fig. 1, gel 4-8). The apparent molecular weight of the monomer increased from 22,000 to 25,000 upon reduction (Fig. 1, gel 6&7). The 45,000 dalton component dissociated to yield the smaller 25,000 dalton product on reduction indicating a dimer/monomer relationship (Fig. 1, gel 5). In ELISA inhibition studies, the 22,000 dalton monomer isolated from bovine GBM yielded an inhibition curve with a similar shape to that of the monomer from human GBM which contains the Goodpasture's antigen (Fig. 2). This bovine component gave 50% inhibition at a protein concentration of 5 µg/ml compared with 0.16 µg/ml for human material, demonstrating crossreactivity and the presence of this antigen in bovine GBM.

In order to test the hypothesis that these collagenase-resistant fragments are contained in the structure of type IV collagen, a procedure was developed for the isolation of type IV collagen from bovine GBM under conditions which preserve its disulfide bonds. The type IV collagen (nonreduced) was then assayed for the presence of Goodpasture's antigen prior to and after collagenase digestion. Bovine GBM was extracted with 8 M urea and the solubilized type IV collagen was purified by gel

exclusion chromatography on Sepharose CL-4B, followed by ion exchange chromatography as previously described (12). The collagen was analyzed by SDS-PAGE (Fig. 3A) before and after reduction of disulfide bonds. Prior to reduction, the collagen exists in the form of very high molecular weight components ($>500,000$ daltons) (gel 1). Upon reduction (gel 2), these components dissociated into four protein bands (α_1 (IV), α_2 (IV), and 164,000, and 152,000 components). The 164,000 and 152,000 components are truncated forms of α_1 (IV) and α_2 (IV) and have been found to occur to varying extents in type IV collagen preparations (12-15). Several additional higher molecular weight components are present in gel 2 (reduced sample). These high molecular weight proteins appear to be multimers of the $\alpha_1\alpha_2$ type IV analogous to those found in other collagen types. This supposition is based on the similarity of the amino acid composition of the nonreduced type IV collagen preparation (Table I) to that reported previously for the monomeric type IV collagen (14) and the fact that aldehyde derived crosslinks are known to be present in the GBM (19).

The presence of the Goodpasture's antigen in the nonreduced type IV collagen preparation was demonstrated by competitive ELISA. Microtiter plates were coated with nonreduced samples of either human Goodpasture's monomer, monomer from bovine GBM, or collagenase-digested (nonreduced) type IV collagen. Collagenase digestion of this type IV collagen preparation yields products (45,000 and 25,000 daltons) which have the same mobilities in SDS-PAGE as those seen in collagenase digests of bovine GBM (data not shown). Binding of the Goodpasture's antibodies was inhibited by the human monomer. Inhibition curves were similar, indicating that all preparations contained equivalent antigens (Fig. 4). In addition binding of Goodpasture's antibodies to coated human antigen was inhibited by nonreduced type IV collagen (Fig. 2). Fifty percent inhibition was observed with a protein concentration of $63 \mu\text{g/ml}$ of intact nonreduced bovine type IV

collagen. The purified monomer from bovine GBM required 5 $\mu\text{g/ml}$ for 50% inhibition suggesting that 8% of the type IV collagen is Goodpasture's antigen. This result compared to a calculated value for α_1 type IV of 15% based on molecular weights from SDS-PAGE (12). Results suggest that the Goodpasture's antigen of the GBM is contained in the type IV collagen molecule. However, this conclusion is ambiguous based on this evidence alone because of uncertainty regarding the chemical nature of the high molecular weight components which are presumed to be multimers of type IV collagen.

In order to provide independent evidence for this conclusion, the structural relationship between the active bovine monomer from GBM and the collagenase-resistant products from $\alpha_{1\&2}$ type IV molecules was investigated. The $\alpha_{1\&2}$ type IV molecules were isolated from bovine GBM which had been reduced and alkylated to dissociate and solubilize them (Fig. 3B) (12). Only $\alpha_{1\&2}$ type IV molecules are present, no multimers could be detected in the preparation even at four times the protein load shown in this figure. The amino acid composition of the preparation is reported in table I and reveals the type IV character of the collagen (12). The $\alpha_{1\&2}$ type IV molecules were next digested with bacterial collagenase under conditions sufficient for total digestion. The digest was dialyzed against water, lyophilized and extracted with 4 M urea/0.2 M sodium phosphate/0.01 M sodium ethylenediaminetetracetic acid. This extraction removed a small amount of low molecular weight peptides and the collagenase. The monomer size digestion product(s) (12) which remained insoluble in this buffer were compared by electrophoresis to: 1) the monomer of bovine GMB (reduced and alkylated), and 2) the monomer, which retains antigenic activity, obtained from bovine GBM (non-reduced) (Fig. 5). Collagenase-resistant products in each of the three samples migrated as doublets upon reduction on this discontinuous gel with apparent molecular weights of 30,500 and 28,500, based on globular protein

standards.

This reveals an identity between the collagenase-resistant products obtained from the purified $\alpha_{1\&2}$ type IV (reduced and alkylated), molecule and the monomer from reduced and alkylated bovine GBM, and that obtained from bovine GBM (nonreduced) which possesses reactivity with the Goodpasture's antibody. The identity is further established by amino acid compositions of the collagenase-resistant products of the $\alpha_{1\&2}$ (IV) molecule and the monomer from bovine GBM containing the reactive antigen (Table I). Both components contain low levels of glycine and 4-hydroxyproline and no detectable hydroxylysine and are therefore noncollagenous, indicating that they were derived from nontriple helical regions of the type IV collagen. Based on these results the Goodpasture's antigen can be localized to the noncollagenous regions of the type IV collagen complex.

The specific reactivity of antibodies from Goodpasture's patients was confirmed by allowing serum from seven patients to react with coated human Goodpasture's antigen, isolated bovine monomer, and collagenase-digested bovine type IV collagen (Fig. 6). Three normal sera, representative of a large population (fifty) of normal sera, showed negligible binding while all Goodpasture's patients sera reacted with all three preparations of antigen.

Based on these studies of the nonreduced type IV collagen complex and the monomeric type IV collagen, we conclude that the Goodpasture's antigen is contained within the type IV collagen of the GBM and that the antigen is localized to the noncollagenous sequences of the molecule. These sequences, with binding activity to Goodpasture's antibodies, can be excised by collagenase digestion of the nonreduced GBM matrix or the purified type IV collagen complex (nonreduced) to yield active fragments. Whether both of the smaller fragments seen on discontinuous SDS-PAGE possess Goodpasture's antigen remains unknown. Upon disulfide bond cleavage, the 45,000 product dissociates to yield the smaller

fragments, indicating a monomer/dimer relationship. The presence of both dimer and monomer suggests that the antigen is incompletely disulfide bonded in the native matrix or that a portion undergoes further cleavage with collagenase to release the monomeric fragments. The ratio of the 30,500 to the 28,500 dalton fragments is nearly identical in the sample derived from the reduced and alkylated type IV to that obtained from the reduced and alkylated bovine GBM but the ratio differs in the sample obtained from nonreduced bovine GBM (Fig. 5). This possibly reflects increased exposure of the collagenase-sensitive sites upon reduction and alkylation of disulfide bonds (22).

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Table 1. Amino acid compositions of purified type IV collagen and of collagenase resistant products of bovine GBM. The analyses were performed on a 6 N HC1/24 h/100°C hydrolysates with a Dionex D-300 analyzer.

Dionex D-300 analyzer.		Collagenase-resistant products		
Amino Acid	Nonreduced type IV collagen	Alpha _{1&2} type IV molecules ^b	alpha _{1&2} molecules ^c	25,000 monomer ^d
residues/1000				
3-Hydroxyproline	11.0	9.9	0	0
4-Hydroxyproline	110.	125.	7.0	2.9
Aspartic Acid	52.2	50.4	67.5	71.9
Threonine	30.0	27.0	65.6	68.9
Serine	47.3	44.9	104.	101.
Glutamic acid	84.9	80.0	91.4	93.5
Proline	69.9	65.4	73.3	72.7
Glycine	278.	290.	95.7	89.6
Alanine	50.2	40.6	78.7	77.5
Half-cystine ^e	8.4	10.3	42.3	35.2
Valine	27.3	25.9	42.0	50.2
Methionine ^f	16.2	22.5	34.0	26.1
Isoleucine	26.4	29.1	42.4	47.8
Leucine	54.6	61.7	74.6	76.3
Tyrosine	10.1	8.7	35.0	39.2
Phenylalanine	29.0	28.6	48.8	42.9
Histidine	9.8	9.4	29.4	32.5
Hydroxylysine	36.4	31.7	0	0
Lysine	11.8	6.5	14.3	17.4
Arginine	37.1	31.7	53.3	55.0
Number of Analyses	3	4	5	2

^aProtein as in figure 3A. ^bProtein as in figure 3B. ^cProtein as in figure 5, gel 4. ^dProtein as in figure 5, gel 5 which had been further purified by reverse phase liquid chromatography. ^eDetermined as carboxymethylcysteine in samples which had been reduced and alkylated. ^fDetermined as the sum of methionine and methionine sulfoxide.

Figure 1. SDS-PAGE of collagenase digests and isolated collagenase-resistant products from nonreduced bovine GBM. Gel 1, protein molecular weight standards: (top to bottom) bovine serum albumin, egg albumin, pepsin, and trypsinogen; Gel 2, collagenase digested bovine GBM; Gel 3, nondigested control bovine-GBM; Gel 4, isolated collagenase-resistant dimer; Gel 5, isolated collagenase-resistant dimer after reduction of disulfide bonds; Gel 6, isolated collagenase-resistant monomer; Gel 7, isolated collagenase-resistant monomer after reduction of disulfide bonds; Gel 8, protein molecular weight standards: (top to bottom) bovine serum albumin, egg albumin, pepsin, trypsinogen, β -lactoglobulin, and lysozyme. Gels 1-3 are 12% acrylamide and gels 4-8 are 7.5% acrylamide (20).



Figure 2. Inhibition of ELISA by nonreduced type IV collagen and monomer isolated from bovine GBM. The binding of Goodpasture's antibodies to human Goodpasture's antigen (26,000 dalton collagenase monomer) could be inhibited by nonreduced type IV collagen (●—●), the isolated bovine monomer (○—○), or the human monomer (□—□). Serum from a Goodpasture's patient was reacted with varying concentrations of the test proteins, any remaining free Goodpasture's antibodies were then detected by ELISA utilizing the human monomer to coat microtiter plates and alkaline phosphatase coupled antihuman IgG (6).

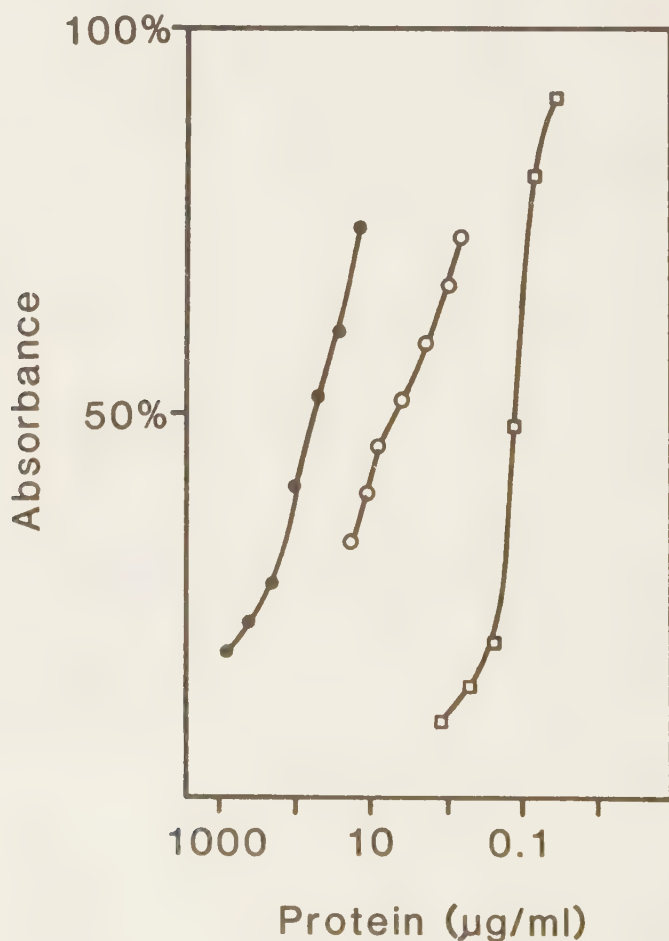


Figure 3. SDS-PAGE of the isolated type IV collagen from bovine GBM. Panel A. Nonreduced type IV collagen isolated from urea-soluble bovine GBM (4% tube SDS-PAGE) (20). Gel 1, type IV collagen electrophoresed without reduction of disulfide bonds; Gel 2, type IV collagen electrophoresed with reduction of disulfide bonds; Gel 3, type I acid soluble collagen. Panel B. Reduced and alkylated α_1, α_2 ; Gel 2, type I acid soluble collagen; Gel 3, globular protein standards: a, myosin; b, β -galactosidase; c, phosphorylase B; d, bovine serum albumin; e, egg albumin; f, carbonic anhydrase. Proteins were detected by staining with comassie blue R.

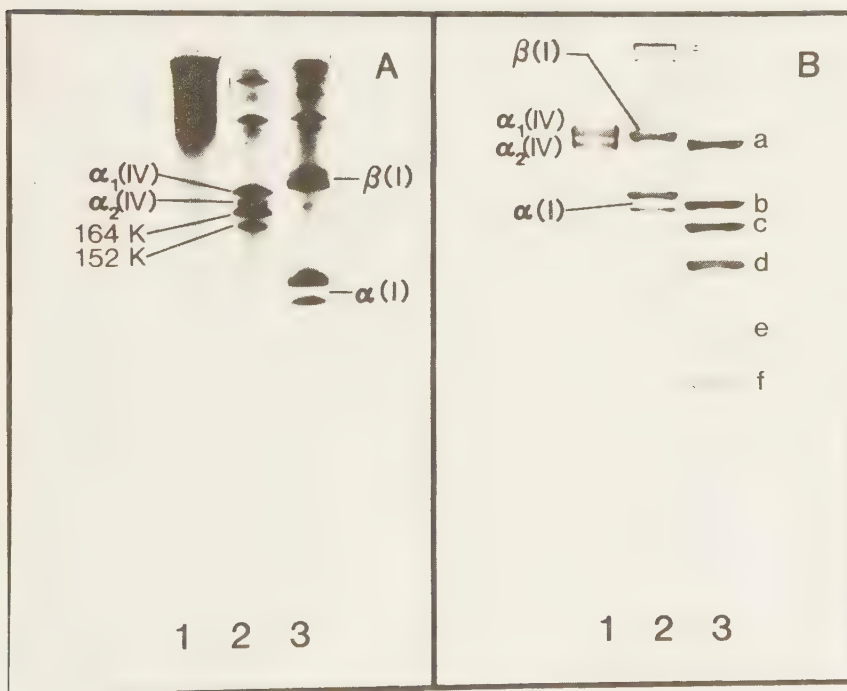


Figure 4. Competitive binding demonstrated by ELISA. The binding of Goodpasture's antibodies to collagenase-digested nonreduced type IV collagen (●—●), the monomer from bovine GBM (□—□), or the human Goodpasture's monomer (○—○), were examined by competitive ELISA (6). The above proteins were coated onto microtiter plates, serum from a patient with Goodpasture's syndrome was mixed with dilutions of the human Goodpasture's antigen and the mixture was allowed to react with the coated proteins. Bound antibodies were detected by incubation with alkaline phosphatase labeled anti-human IgG.

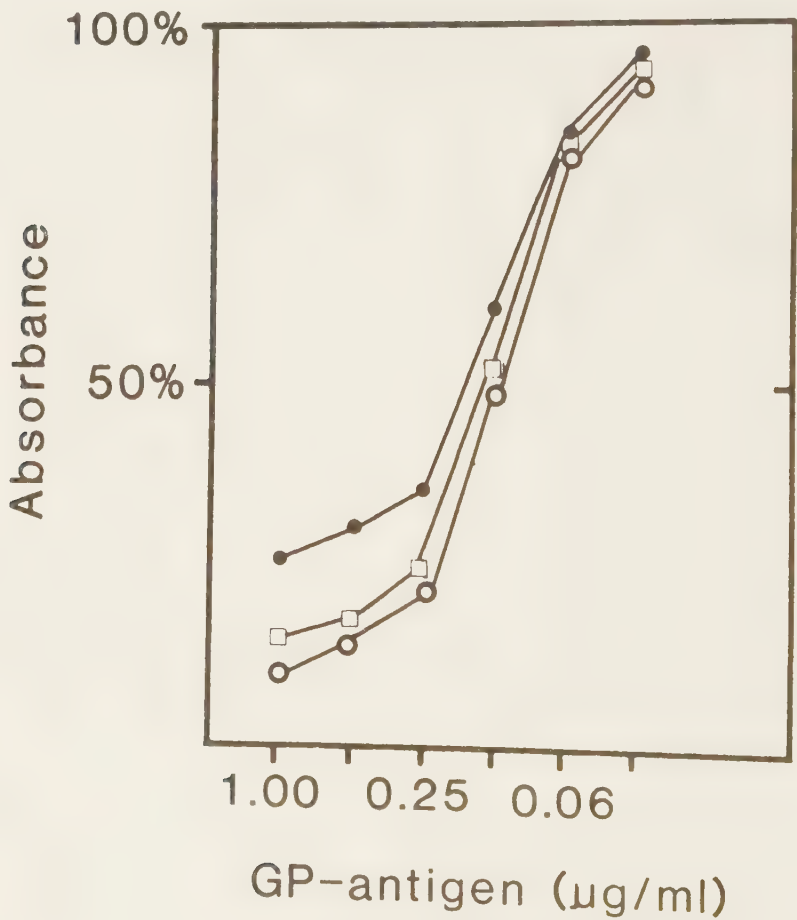


Figure 5. SDS-PAGE comparing collagenase digestion products of bovine GBM, reduced and alkylated bovine GBM, and reduced and alkylated $\alpha_{1\&2}$ type IV collagen. Samples were applied for electrophoresis to a 4-22% acrylamide gradient slab SDS-PAGE (21); lanes 1&2, globular protein standards: a, β -galactosidase; b, phosphorylase B; c, bovine serumalbumin; d, egg albumin; e, glyceraldehyde-3-phosphate dehydrogenase; f, carbonic anhydrase; g, trypsinogen; h, trypsin inhibitor; i, α -lactalbumin; lane 3, reduced and alkylated $\alpha_{1\&2}$ type IV prior to collagenase digestion; lane 4, same as lane 3 after collagenase digestion; lane 5, 30,000 molecular weight collagenase digestion product isolated from bovine GBM, reduced; lane 6, same as lane 5; lane 7, 30,000 molecular weight digestion product isolated from reduced and alkylated bovine GBM.

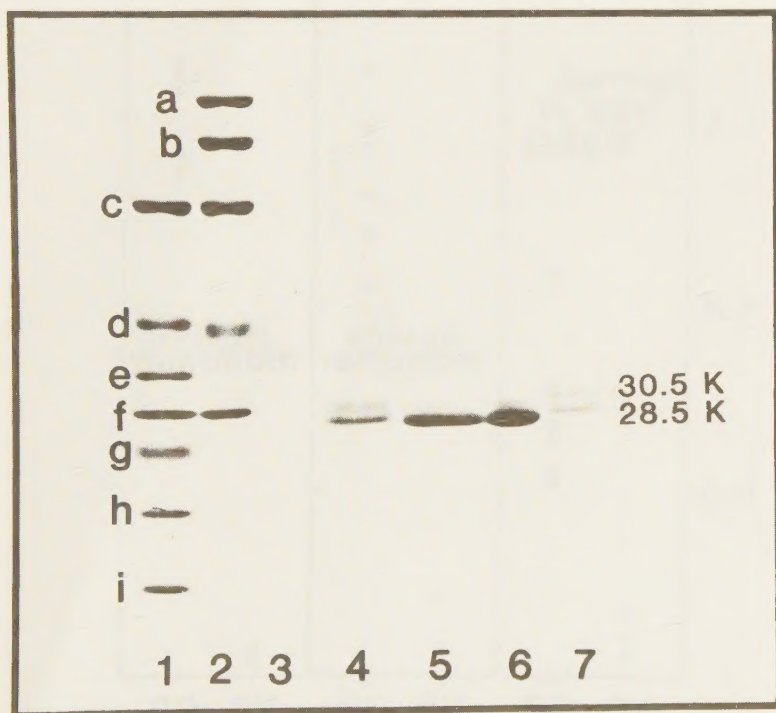


Figure 6. ELISA demonstrating the reactivity of serum antibodies from seven patients with Goodpasture's syndrome and three normal control sera. Human Goodpasture's monomer (26,000 dalton collagenase product), monomer from bovine GBM, and collagenase digested nonreduced type IV collagen from bovine GBM were coated to microtiter plates. A 1/25 dilution of the sera were incubated for 1 hour and bound IgG was detected by alkaline phosphatase conjugated IgG (6).

